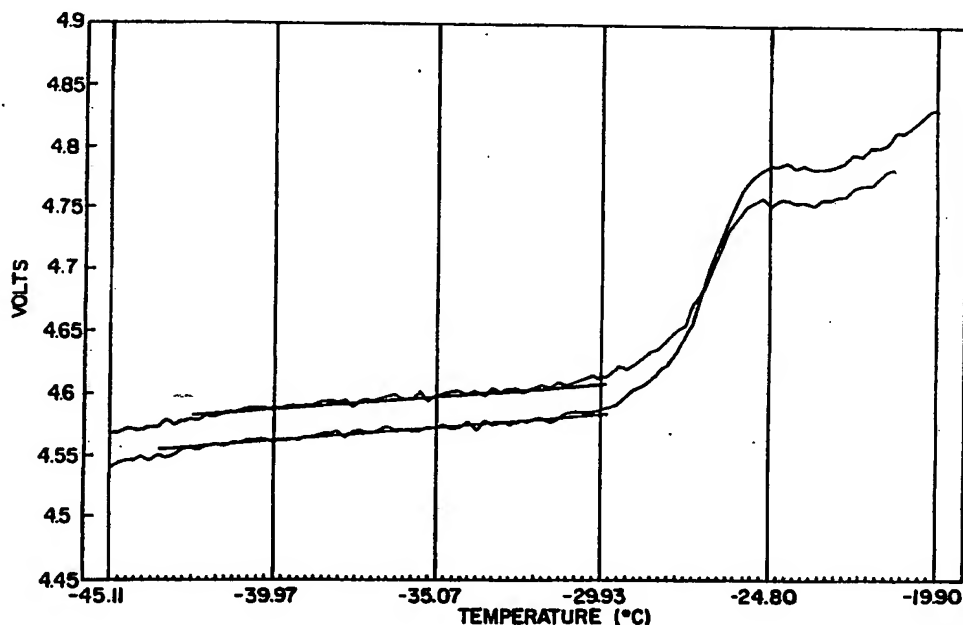




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 5/00, A01N 1/02		A1	(11) International Publication Number: WO 93/14191
			(43) International Publication Date: 22 July 1993 (22.07.93)
(21) International Application Number: PCT/US93/00249		(74) Agents: KENNEY, J., Ernest; Bacon & Thomas, 625 Slaters Lane, Fourth Floor, Alexandria, VA 22314 (US) et al.	
(22) International Filing Date: 21 January 1993 (21.01.93)			
(30) Priority data: 824,116 21 January 1992 (21.01.92) - US		(81) Designated States: AU, CA, FI, JP, NO, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(71) Applicant: CRYOPHARM CORPORATION [US/US]; 2585 Nina Street, Pasadena, CA 91107 (US).		Published With international search report.	
(72) Inventors: GOODRICH, Raymond, P., Jr. ; 140 S. Mentor, #312, Pasadena, CA 91106 (US). COKER, Samuel, O., S. ; 1800 State Street, #54, South Pasadena, CA 91030 (US). ARNAUD, Franciose, G. ; 610 East California, #1, Pasadena, CA 91106 (US). HACKETT, Roger, W. ; 2046 Monte Vista Street, Pasadena, CA 91107 (US).			

(54) Title: METHOD OF FREEZING CELLS AND CELL-LIKE MATERIALS



(57) Abstract

A process is provided for freezing, including freeze-drying of cells, cell-membranes or cell-like materials using a cryoprotectant medium which stabilizes the cells or membranes for freezing or freeze-drying and allows for freezing or freeze-drying to be performed at -60 °C or higher.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SK	Slovak Republic
CI	Côte d'Ivoire	LJ	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	MC	Monaco	TG	Togo
DE	Germany	MG	Madagascar	UA	Ukraine
DK	Denmark	ML	Mali	US	United States of America
ES	Spain	MN	Mongolia	VN	Viet Nam
FI	Finland				

**METHOD OF FREEZING CELLS AND
CELL-LIKE MATERIALS**

FIELD OF THE INVENTION

The present invention is directed to a method for
5. freezing, including freeze-drying, cells or cell-like
materials. In particular, the invention is directed
to a method for freezing platelets for storage.

BACKGROUND OF THE INVENTION

Current storage of fresh human platelets in liquid
10 form (i.e., as platelet-rich concentrate in residual
plasma) is limited in the following ways:

- 1) Platelets in liquid suspension (platelet
concentrate) are generally not stored refrigerated as
they suffer from a well-documented "storage lesion"
15 involving loss of clotting function and in vivo
viability (refer to AABB Technical Manual, 10th
edition, 1990, pp.51-52). Platelet concentrates
stored refrigerated are limited to a 72 hour shelf
life due to the rapidity of the storage decay. The
20 method used by most blood banks is to store platelet
concentrate at room temperature (20-24 deg. C) with
continuous agitation by mechanical rocking.
Agitation is essential at these temperatures to
prevent cell aggregate formation. Currently

SUBSTITUTE SHEET

regulations of the U.S. Food and Drug Administration (FDA) limit room temperature storage to 5 days.

2) The quality of room temperature stored platelet concentrates, as measured by aggregometry and other in vitro measures, decays after the first 24 hours of storage to some 75-85% of fresh values. Further deterioration occurs by five days of storage, such that the therapeutic value is only about 30% of fresh cells (i.e., the clotting efficacy of infused 5 day-old concentrate in patients, as measured by their bleeding time reduction, is only some 30% of a comparable dose of fresh platelets).

3) FDA limits room temperature storage of platelet concentrates to five days due to the threat of bacterial growth in the nutrient rich plasma concentrate. Contamination of platelet concentrates by Yersinia bacteria has been associated with deaths due to platelet transfusion.

4) The short shelf life means that some 25% of platelet concentrates collected by U.S. blood banks become unusable by outdating. This is a loss of a valuable voluntary resource.

The current useful method of storage of platelet concentrates in frozen form uses a 5% DMSO storage method developed by Schiffer et al (1983) Annals N.Y. Acad. Sci. 411, 161-169. This requires that the cells be stored at - 120 deg. C in the vapor phase of liquid nitrogen. Most blood banks do not use this method due to the expense of liquid nitrogen storage, plus the impracticality of shipping in liquid nitrogen. (See AABB Technical Manual, 1990, pp. 100-101 for review of blood cell freezing literature). Valeri has reported a 6% DMSO method that allows

SUBSTITUTE SHEET

storage at temperatures as high as -65 deg. C,
(referenced in the AABB Manual p. 102), a temperature
compatible with ultra-low mechanical freezers. These
freezers are also very expensive and have limited
5 storage capacity.

An accepted shelf life for frozen platelets
in 5% DMSO is 3 years. In actual clinical use, a
frozen-thawed DMSO unit of platelets will normally
exhibit about 50-60% of the clotting efficacy seen
10 with a fresh platelet concentrate unit (i.e., usually
two units of frozen thawed platelets are needed to
achieve the same effect in vivo as one fresh unit).

It should be noted that FDA has not approved any
DMSO platelet frozen storage method due to
15 controversy over the efficacy and possible toxicity
of DMSO (usually the frozen cells are thawed and
infused directly, along with the 5% DMSO). Thus,
U.S. blood banks today only freeze and infuse
platelets with the approval of their medical review
20 boards (i.e., interstate transport is prohibited).

Platelets are collected at most blood collection
facilities, ranging from large regional blood centers
(over 100,000 donations per year) to local hospital
blood banks. Platelets can only be stored for 5 days
25 as a platelet-rich plasma fraction at room
temperature according to current FDA regulations.
Although platelets can be frozen at -80 degrees for
up to two years, the expense of freezing has
discouraged frozen storage among civilian entities.
30 The short shelf life is a major impediment to
directed pre-deposit of plateletpheresis units.

A second issue problem is the transport of frozen
platelets, which presents difficulties in container

SUBSTITUTE SHEET

design, especially for air shipment. There is thus a need for lyophilized platelets which can be stored, shipped and reconstituted for therapeutic use.

Platelets play a vital role in blood clot formation and platelet transfusions are administered to arrest an existing bleeding condition or to prevent an imminent loss of blood. Often platelet transfusions are indicated when the patient cannot mobilize his own platelet reserves, as a result of standard chemotherapy treatments or diseases that cause low levels of circulating platelets or loss of normal platelet function. The American Red Cross (ARC) in 1987 reported a total of 4,500,000 transfusions of random donor platelet units and 350,000 transfusions of plateletpheresis units during calendar year 1986 in the United States. Some 245,000 patients received platelet transfusions during this period. The average patient receiving random donor units isolated from whole blood donations required 6 platelet units per transfusion event and also required 6 separate transfusions per year. The high rate of transfusions reflects the need for multiple transfusions for cancer patients undergoing aggressive chemotherapy or bone marrow transplantation as an adjunct to chemotherapy.

Multiple platelet transfusions can cause immune sensitization and rejection, especially when random donor units are used. This sensitization reaction, combined with improved plateletpheresis methods, has spurred demand for directed-donor plateletpheresis units. Plateletpheresis selectively isolates platelets from whole blood and returns the depleted blood to the donor's arm. This allows collection of concentrated platelet units from a single donor, as often as once per week. Each plateletpheresis unit

SUBSTITUTE SHEET

contains the equivalent of 5-6 units obtained from donated whole blood (random donor units).

Plateletpheresis allows more closely matched donors, such as close relatives, to donate and minimize the

5 risk of sensitization. The use of concentrated plateletpheresis units also reduces the patient's exposure to numerous random donors, which reduces both sensitization risk and exposure to viral
contaminants (a chemotherapy patient may require 60
10 platelet units during the course of treatment, and may be exposed to over 50 random donors).

Platelets are prescribed to control massive bleeding events before or during cardiopulmonary bypass surgery, and organ transplants. Unlike red cells,

15 the normal human body possesses platelet reserves that can be mobilized rapidly if blood is lost. As a result, the U.S. Department of Health and Human Services advises that platelets not be wasted on prophylactic transfusions for routine surgery or even
20 most trauma situations (patients can cope with a major bleed of 20 units by mobilizing platelets provided red blood cells are administered).

Bone marrow transplants designed to restore marrow destroyed by chemotherapy accounts for the single
25 most intensive use of platelet transfusions. Most patients recovering from chemotherapy require at least three weeks (and sometimes months) before the new bone marrow graft can begin to produce platelets.

During this period circulating platelet levels are
30 maintained by transfusions, which must be frequent due to the short 5 day circulation lifetime of normal platelets.

The present invention provides a method utilizing a buffer for platelets which allows frozen storage at

SUBSTITUTE SHEET

high temperatures (i.e., in the range of -20 deg. C). This temperature range is easily achieved by conventional mechanical freezers such as chest freezers now used for food storage. The present invention enables long term frozen storage, which will obviate the short shelf life of room temperature or refrigerated storage, as well as the threat of bacterial growth in room temperature stored liquid concentrates. The prohibitive costs of liquid nitrogen frozen storage or ultra-low temperature mechanical freezers are also eliminated, and the use of DMSO is eliminated.

The present invention provides a method for freezing (and freeze-drying) cells or cell-like materials, including platelets, by the use of polymer glass transition theory to determine an effective cryopreservation for natural cells suspended in an aqueous environment. As reviewed by Levine and Slade (1987) Water Science Reviews (F. Franks, ed.) Vol. 3, pp. 79-185, Cambridge University Press, in aqueous systems comprising many natural and synthetic polymers, water acts as a plasticizer that affects the glass-to-rubber transition temperatures (T_g') of the aqueous-polymer system. This area of research has had a significant impact on the design of polymeric food materials (such as those based on starches), since the raising or lowering of T_g' can confer unique advantages for processing or storage stability of a material.

A working definition of a glass (Levine and Slade, 1987) is that it comprises a mechanical solid capable of resisting flow. In a typical amorphous glass the viscosity is extremely high, in the range of 10^{11} to 10^{14} Pa-s at the glass transition temperature (T_g). The T_g value, as illustrated in Figure 2.4 of Slade

SUBSTITUTE SHEET

and Levine (1987) occurs at the transition from the glassy fluid to the rubbery fluid state for systems comprising glassy or partially crystalline polymers. Below T_g , in the glass phase, the high viscosities preclude molecular diffusion and hence chemical reactions (which can lead to product spoilage) cannot proceed at significant rates. From this same figure another important point can be seen: that once the product temperature exceeds T_g , viscous flow can occur in the rubbery state, allowing for molecular diffusion and chemical reaction rates to proceed with exponential kinetics (WLF kinetics).

As a practical example, this explains why certain frozen and perishable biological products, such as human blood cells, must be stored within carefully defined temperature ranges (American Association of Blood Banks Technical Manual, 10th edition, 1990, chapter 5, pp. 91-103). Currently accepted blood cell freezing solutions containing glycerol or dimethylsulfoxide (DMSO) form glasses at very low temperatures (between -65°C and -198°C), thereby maintaining the cells in a chemically unreactive environment. However, exposure to temperatures that exceed T_g lead to poor cell viability as measured by red blood cell circulation time following freeze-thawing and transfusion or bleeding time reduction following transfusion of frozen-thawed platelets. In essence, the perishable cells can spoil even at temperatures below 0°C normally thought of as "freezing temperatures" because the cryoprotective media have T_g values much lower than the -20°C range commonly achieved by standard kitchen freezers.

Many of the fundamental principles of glass transition theory have derived from physical chemical studies in single component systems (see Levine and

SUBSTITUTE SHEET

Slade, 1987) capable of forming a homogeneous "pure" glassy phase at the glass transition temperature (T_g) characteristic of that component, and in multicomponent systems. The present invention, however, provides multi-component aqueous cryopreservative systems that at appropriate temperatures form partially crystalline mixtures of water ice with interspersed regions of a separate amorphous glass phase. As defined in Pikal (1990), BioPharm, Sep-Oct 1990, the glass transition temperature of the amorphous phase in a partially crystalline aqueous system will be defined as T_g' , to distinguish this particular phase from the glass transition temperatures of individual buffer components (i.e., the component T_g values).

SUMMARY OF THE INVENTION

The present invention provides a method, and cryoprotective compositions for use therewith, for freezing, including freeze-drying, cells or cell-like materials. The cells and cell-like materials include erythrocytes, platelets, cell-membranes, stem cells, lymphocytes, and the like. The invention provides cryoprotective compositions which protect the cells or cell-like materials during freezing, freeze-drying and/or storage and which permit these processes and storage to be performed at temperatures which do not require liquid nitrogen, potentially toxic materials such as DMSO, or expensive low temperature refrigeration equipment. Typically, according to the present invention, freezing or freeze-drying may be performed above about -45°C to about room temperature, and storage conditions may be from -80°C to 0°C (for frozen cells or cell-like materials), or up to room temperature (for lyophilized cells or cell-like materials). Predetermined temperatures for freezing, freeze-drying and storage of the cells

SUBSTITUTE SHEET

which allow for optimum cell characteristics or recovery and shelf-life may be obtained by utilization of cryoprotective compositions according to the invention.

- 5 The aqueous mixture of cells, cell membrane or cell-like materials will be characterized by a high glass transition temperature, usually above about -60°C, and will contain a cryoprotective component. This component will comprise one or more permeants, 10 i.e., compounds capable of penetrating the cells or cell membranes. The cryoprotective components will also contain one or more impermeants, i.e., compounds impermeable to the cells or cell membranes, wherein the amount of impermeants present in the mixture is a 15 weight fraction sufficient to overcome the predicted depression in the glass transition temperature of the mixture attributable to the permeants.

BRIEF DESCRIPTION OF THE FIGURES

- FIG. 1 is a phase diagram of an aqueous-polymer 20 solution.

FIG. 2 is the profile of a DSC thermal analysis of a buffer described in Example 1.

FIG. 3 is an osmoscan profile for rehydrated red cells described in Example 6.

- 25 FIG. 4 is a plot to glass transition temperature as a function of drying time of a buffered red blood cell mixture described in Example 3.

FIG. 5 is a plot of glass transition temperature as a function of residual moisture of a buffered red 30 blood cell mixture described in Example 3.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

- Among the polymeric food ingredients that have been studied by others are natural polymers such as gelatin, gluten, starches, and frozen aqueous 35 solutions of small sugars (i.e., mono- and

SUBSTITUTE SHEET

disaccharides), polyols (glycerol), and starch hydrolysis products. Synthetic polymers such as PVP (polyvinylpyrrolidone) and PVAc (polyvinylacetate) in water have also been studied. Each of these materials in aqueous solution can be considered as an amorphous or partially crystalline polymer system that is plasticized by water. Glass transition theory as applied to these partially crystalline water-polymer systems focuses on the critical variables of moisture content (or percent dryness), temperature, and time to predict and regulate the cryostabilization of frozen or freeze dried products. The present invention provides aqueous-polymer buffers for the cryostabilization of natural cells by application of these principles.

Referring to Figure 1 an idealized phase diagram is shown for an aqueous-polymer solution comprising an amorphous, partially crystalline system, which illustrates the relationship between the moisture content (or percent solids) and temperature. The hypothetical system is characterized by distinct freezing and glass transition (T_g) curves that define different physical states: R = rubbery phase, SOL = solution phase, ICE/SOL = mixture of ice and solution, and G = glassy phase. The essential principle is that when the system is maintained at temperatures below the glass transition curve, an amorphous (noncrystalline) glass exists that is characterized by extremely high viscosities that prevent molecular diffusion on practical time scales. Thus, in the glassy state most chemical reactions such as degradative oxidation reactions that can damage cellular components, are inhibited. The slow kinetics of chemical reactions under these conditions lead to a cryostabilized product at the appropriate temperatures.

SUBSTITUTE SHEET

From Figure 1 it can also be seen that T_g' increases most rapidly in aqueous glassy polymer systems of low moisture content. This is also expressed as a depression of T_g' by the plasticizing effect of increasing water content. PVP represents a typical water-soluble, glassy (non-crystallizable) polymer. Therefore, reduction of water content (i.e., by freeze-drying) is one approach to elevated temperature storage stability, as the resulting dried polymer composition has a higher glass transition temperature. It can be seen from our hypothetical curve in Figure 1 for a water-PVP system, that the T_g' can exceed 0 degrees Celsius at sufficiently low moisture content. This indicates that prolonged storage at above freezing temperatures can be achieved under the proper conditions.

It is known that T_g for a pure polymer varies with the polymer weight average molecular weight: T_g increases with increasing average molecular weight, up to a limiting molecular weight value. The greatest increase in glass transition temperature is seen at low moisture contents (i.e., high weight percent of polymer). Thus, selection of water-soluble polymers of increasing average molecular weight to incorporate into an aqueous cryostabilizing buffer can further elevate the T_g' of the amorphous phase, due to the increased T_g of the individual polymer component. The relative contribution of each polymer component to the overall T_g' increases as the weight percent of that component in the aqueous solution increases.

Furthermore, by increasing the weight fraction of starch, or conversely increasing the weight fraction of water will respectively increase or depress the T_g' of the system. It can thus be extrapolated that

SUBSTITUTE SHEET

more refined control of the T_g' value for a particular aqueous cryostabilizing buffer system can be attained by adding to pure water (which has a t_g of -135°C) multiple solute components of differing molecular weights (and hence higher or lower component T_g values) in appropriate weight percentages.

According to the invention these considerations of individual component T_g values and the weight percent of each component in an aqueous are utilized to provide multicomponent buffer formulations that have elevated (above -45°C) T_g' values. The calculated buffer T_g' values, based on known component T_g values and their weight percent in a particular buffer formulation, allow accurate predictions of the buffer T_g' .

For freeze-drying a cryostabilizing buffer must have a T_g' that is appreciably above -45°C , since the product cannot be kept frozen at lower temperatures if efficient sublimation is to occur. Below this temperature the vapor pressure of water is so low that even under high vacuums of 100 mTorr or less achieved in conventional freeze-drying equipment the rate of sublimation is too low to be practical.

The phenomenon of freeze concentration during the freezing process is another consideration. In aqueous systems containing dissolved solutes, the initial freezing event involves conversion of liquid water to crystalline ice. The dissolved solutes are thereby concentrated out of the ice phase into a residual liquid phase in which the concentration of dissolved solutes increases significantly. At sufficiently low temperatures, below T_g' , this non-crystalline phase becomes a glass. Implicit in this

SUBSTITUTE SHEET

description is the fact that valuable components such as proteins or blood cells also become concentrated into the glassy phase. The high electrolyte and buffer concentrations in this phase can be damaging to proteins or cellular membranes. Hence, it is preferable to start with the minimal acceptable concentrations of salts and buffering agents (buffered phosphate), as these will become further concentrated during freezing into the same amorphous phase that contains the product of interest.

The process of lyophilization by sublimation is defined as removal of water vapor directly from the ice phase. During lyophilization the ice crystals are removed by sublimation, leaving open pores or channels in the frozen, partly dried matrix. Formation of these channels is critical for thorough primary drying (in which the bulk of the water is removed as water vapor from the ice phase), as these channels allow water vapor to escape from regions below the surface of the product "cake". During primary drying the product should be maintained below the collapse temperature, which is usually identical to the T_g' of the amorphous glass phase containing regions of maximally concentrated solutes. If the product temperature exceeds T_g' during primary drying, then regions of amorphous glass will have sufficient fluidity to undergo viscous flow once the ice is sublimed, thereby blocking the open channels and preventing further sublimation. Thus, it can be seen that cryostabilizing buffers must first achieve a measurable and elevated T_g' value that will allow the material to be held below T_g' during ice sublimation.

Once the bulk of the water in the product is removed during primary drying, the secondary drying

SUBSTITUTE SHEET

phyase begins. During secondary drying, the residual moisture in the amorphous phase is removed by evaporative or vacuum drying. Evaporative drying involves removal of water vapor from the nonicy
5 state.

The accepted methods for frozen storage of human blood cells (red cells, platelets, stem cells and lymphocytes) generally involve aqueous glycerol or DMSO solutions. Both of these compounds have very
10 low glass transition temperatures (T_g values), such that the current formulations for cell freezing (40% w/v glycerol or 5% v/v DMSO; see the AABB Technical Manual, 1990) have low glass transition temperatures that are below about -65°C (in these systems the
15 aqueous solution is referred to as having a T_g value, since the term ' T_g ' is meant to specify glass transition temperature in a partially crystalline system). In addition, both glycerol and DMSO are
20 potent chaotropic agents that prevent the formation of crystalline ice in aqueous solutions when present in the amounts currently used. Although this prevents ice crystal damage to cells during frozen storage at ultralow temperatures, the requirement of ice channel formation during freeze-drying suggests
25 that these cryoprotectant formulations are not useful for freeze-drying by sublimation, or for freeze-evaporative drying because of the need for very low frozen temperatures at which the vapor pressure of water is very low. This also explains the empirical
30 findings reviewed in the AABB Technical Manual (1990), chapter 5, that cells stored frozen in either of these solutions lose their functional viability if the storage temperature exceeds -65°C for the high glycerol solution, or -120°C for the DMSO solution:
35 frozen storage at temperatures that exceed T_g lead to a glass-to-rubber transition in which viscous flow

SUBSTITUTE SHEET

occurs in the rubbery state, allowing for molecular diffusion and degradative chemical reactions to proceed at appreciable rates. Neither the existing 40% glycerol system or the DMSO system are useful for high temperature frozen storage (in the range of -20°C), but this temperature range is a necessity for efficient lyophilization.

The present invention provides aqueous buffer solutions for cryostabilization of cells and cell-like materials that comprise buffers having elevated glass transition temperatures (T_g'). The higher the glass transition temperature for a particular aqueous suspension of biological cells in a buffered polymer mixture, the higher the useful storage temperature.

From Figure 1 it can be seen that the glass transition curve for any aqueous polymer system depends upon the weight fraction of total solids in the solution. Based on the foregoing discussion, the following considerations should be used in the selection of an aqueous cryostabilizing buffer system according to the invention for maintaining perishable biological materials, such as cells or proteins, in a stable frozen state (either for frozen storage or during a freeze-drying procedure):

1) The buffer should have the highest practical glass transition curve (T_g'), with a minimal component of salts or buffering agents that may contribute to freeze-concentrate damage.

2) The overall glass transition temperature of a particular aqueous buffer system will depend on the glass transition temperatures of each of the individual solutes and upon their respective weight fraction in the mixture. This relationship can be approximated by the following:

SUBSTITUTE SHEET

Equation 1.

$$Tg'(\text{buffer}) = (TgA)(Wt.\%A) + (TgB) \dots + (Wt.\%B) + (TgN) \\ (Wt.\%N) - 5.2 \text{ } ^\circ\text{C}$$

5 where $Tg'(\text{buffer})$ is the glass transition
temperature of the total solution; $Tg A$ is
the glass transition temperature of solute A;
Wt. % A is the weight fraction percent of
solute A in the buffer; N is the last member
10 of N components; and
5.2 $^\circ\text{C}$ is an empirically derived value to
correct for volume displacement of the solid
components in the solution.

3) For use with biological or artificial
membrane systems (i.e., cells or liposomes), the
15 buffer should contain permeant cryoprotectant agents
capable of penetrating the phospholipid bilayer of
the membrane to stabilize the internal lipid bilayer,
and also to stabilize intracellular or intravesicle
components (organelles, internal membranes, enzymes,
20 etc.). Useful membrane-permeants include small
sugars, such as monosaccharides, and polyalcohols
(polyols) such as glycerol or polypropylene glycol.
These small sugars and polyols generally have low
individual glass transition temperatures, and their
25 inclusion in a buffering system will lower the
overall Tg' of the solution proportional to their
weight fraction in the solution, as described in
Equation 1 above. These same polyhydroxy compounds
also serve as water replacement agents as suggested
30 by Crowe (Bio Chem. Biophys. Acta, 939, 327-334
(1988)), to produce the structure of cell components,
such as the phospholipid bilayer.

SUBSTITUTE SHEET

- 4) To achieve elevated glass transition temperatures for aqueous buffers comprising membrane permeant small sugars and/or polyols, one or more water soluble polymers must be included in sufficient weight fraction percentages to overcome the Tg-depression caused by the cell permeant components. Use of higher average molecular weight polymers is helpful as Tg for an amorphous polymer increases with molecular weight. These high molecular weight polymers can comprise natural (starches, gelatins, etc. derived from plant, animal or microbial sources) or synthetic (PVP) polymers, or mixtures of polymers of various average molecular weights.
- 5) For use in transfusion medicine, the components of the aqueous buffer system must also be biocompatible, nontoxic, and available as sterile, pyrogen-free materials.

Using Equation 1 above, buffers are provided based on the calculated glass transition temperature, as shown, for example, in Table 1. Equation 1 shows that control over the Tg' of an aqueous cryostabilizing buffer system can be achieved by exploiting the glass transition temperature characteristic of each individual buffer component and its weight fraction in the buffer solution.

Preferred impermeant compounds include amorphous and/or partially crystalline polymers or mixtures thereof. These may be natural or synthetic polymers, preferably of molecular weight over about 300, and most preferably about 10,000-500,000. The polymers are preferably present in the solution in weight percentages from about 1% to their limit of solubility. Preferred weight fractions are 5%-25%.

Preferred permeant compounds include carbohydrates, preferably monosaccharides, disaccharides or mixtures thereof. Typically each of the permeants will be present in a weight fraction
5 from about 0.01% to their respective solubility limits. Preferred weight fractions are from about 0.5% to 20%.

The preferred monosaccharides are 6-carbon hexose sugars and 5-carbon pentose sugars. Preferred
10 disaccharides are maltose, lactose, sucrose, trehalose.

Polyalcohols may also be used as permeants, preferably in concentrations of 0.1% to 40% by volume, and most preferably in the range of 5%-25%.

15 In Table I below, the reported glass transition temperatures for a series of useful buffer components are shown. These values are reported in Slade and Levine (1987), pp. 112-115.

SUBSTITUTE SHEET

Table I.

REPORTED GLASS TRANSITION TEMPERATURE VALUES.

5	<u>Starch Hydrolysis Products</u>	<u>Manufacturer</u>	<u>Tg' (deg. C)</u>
	Paselli MD- 10 (potato)	AVEBE	-8
	Paselli SA- 10 (potato)	AVEBE	-9.5
	Maltrin M150 (corn)	GPC	-13.5
10	<u>Polyalcohols</u>	<u>Molec. Wt.</u>	<u>Tg' (deg. C)</u>
	ethylene glycol	62.1	-85
	1,3-butanediol	90.1	-63.5
	glycerol	92.1	-65@
20	<u>Sugars (Mono/Di saccharides)</u>	<u>Molec. Wt.</u>	<u>Tg' (deg. C)</u>
	xylose	150.1	-48
	ribose	150.1	-47
	glucose	180.2	-43
	fructose	180.2	-42
	galactose	180.2	-41.5
	mannose	180.2	-41
	sucrose	342.3	-32
	maltose	342.3	-29.5
	trehalose	342.3	-29.5
	lactose	342.3	-28

SUBSTITUTE SHEET

	<u>Polymers</u>	<u>Approx. Average MW</u>	<u>Tg'</u> <u>(deg.</u> <u>C) *</u>
	PEG	300	-63.5
5	gelatin	N.D.	-8
	dextran	9,400	-13.5
	hydroxyethyl starch (HES)	N.D.	-6.5(1)
	PVP-10	10,000	-26
	PVP-40	40,000	-20.5

10 PEG = polyethylene glycol
PVP = polyvinylpyrrolidone

Pure water has an estimated Tg of - 135°C.

* The reported polymer Tg values in Slade and Levine (1987), p. 131, are compiled from literature values
15 (the value for gelatin is based on collapse temperature during freeze-drying).

(1) No average molecular weight value is reported in Slade and Levine for HES, and this value will vary depending on the average MW (HES is available in a range of molecular weights, up to about 500,000).
20 @ Pure glycerol has a Tg of -95°C.

From the representative Tg' values presented in Table I, it and the teachings herein on these kinds of components, added in different weight fraction
25 proportions, can be used to control the final Tg' of an aqueous buffered medium. Use of multicomponent buffers containing low molecular weight, membrane-permeable molecules (polyalcohols and monosaccharides) are vital for preservation of
30 intracellular structures. As seen from Table I, these small molecules have low individual Tg' values, and if present in large concentrations would lead to excessively low buffer Tg' values (i.e., the accepted 40% glycerol medium for freezing red cells has a low
35 glass transition due to the -65°C glass transition of the glycerol component). Thus, these small molecules should preferably be used in low concentrations if a high Tg' buffer is desired. In addition, these small

SUBSTITUTE SHEET

molecules can be formulated in combination with one or more polymers of varying average molecular weights to raise the overall T_g' of the buffer. By these strategies a suitable cellular cryostabilizing buffer for use under a range of freezing temperatures (and ambient temperatures in the freeze-drying process) can be formulated.

Other buffer parameters, such as potential cell toxicity, cell aggregation behavior, or cellular cryostabilizing efficacy, under the desired freezing or freeze-drying conditions may also be considered in selecting components of the buffer. Different buffer components may be required for specific mammalian cell types; for example, in the present invention we show that glycerol is amenable to human red blood cell cryopreservation, but is not as efficacious with human platelets. Hence the method of the present invention focuses buffer design in terms of the physical-chemical requirements of freezing or freeze-drying, but each mammalian or other cell type may have special requirements based on its unique differentiated properties (i.e., the need to preserve oxyhemoglobin in mature red blood cells or the need to preserve nuclear structures in nucleated cells).

The calculated buffer T_g' value can be directly compared to the measured value determined by thermal analysis by differential scanning calorimetry (DSC) analysis. A representative DSC profile for one aqueous buffer formulation is shown in Figure 2, for a three component buffer containing as its principle weight fraction components 5% mannose/10% lactose/20% PVP-40,000 MW (trace buffer salts are present but are ignored for the purpose of calculating T_g' as their contribution is minimal). The calculated T_g' derived from Equation 1 for this formulation is -

SUBSTITUTE SHEET

30.7°C, and in Figure 2 the DSC traces for two sample runs show the measured values to be -30.0°C and -30.25°C. These values are in excellent agreement with the calculated Tg' value. It should be noted
5 that in practice this cryostabilizing buffer was diluted in the ratio 2.3 parts buffer: 1 part cell suspension, so in Figure 2 the DSC analysis was performed on a 2.3:1 mixture of buffer:water to approximate final concentrations in actual use.

10 It will be understood that the buffers according to the present invention may also contain basic salts compatible with the biological material. Such salts include electrolytes, capacitors, metal cations, metabolites, buffer salts, vitamins, hormones, growth
15 factors, and the like. The cells, cell-membranes and cell-like materials which may be treated according to the present invention include mamalian cells (including human), tieeues, peripheral blood cells (red blood cells, platelets, stem cells, leucocytes),
20 mammalian cell membranes, including membranes of periferal blood cells.

From the foregoing description one of ordinary skill in the art may formulate cryostabilizing buffers for use in frozen storage or for use in
25 freeze-drying of perishable biological cells or proteins.

Examples

Example 1

For frozen storage stability, we formulated several
30 three component buffers listed in Table 2, following the main requirements: 1) the buffer must include at least one membrane permeant cryoprotectant, selected

SUBSTITUTE SHEET

from available monosaccharides that can traverse phospholipid bilayers (polyalcohols may also be used); 2) the buffer may include a membrane impermeant cryoprotectant, usually a disaccharide or higher molecular weight carbohydrate or polymer; 3) at least one polymer must be present in sufficient weight percentage and having sufficiently high average molecular weight (hence high T_g) to boost the glass transition temperature (T_g') of the buffer solution; and 4) the relative weight percentages of the individual buffer solutes must be adjusted so that the calculated T_g' exceeds -45°C , and preferably for convenient frozen storage, is above -25°C (buffers prepared to have a calculated T_g' are then measured by DSC analysis to confirm the actual T_g' at final buffer concentrations contemplated for use with cell suspensions); 5) the use of polyhydroxy components (monosaccharides, disaccharides, polyalcohols, starches) contributes to membrane stabilization by water replacement.

In Figure 2 we show a typical DSC thermal analysis profile obtained from a sample of buffer #1(5% mannose/10% lactose/20% PVP-40) in Table 2. The buffer:water mixture exhibits a characteristic and reproducible inflection point at the T_g' onset temperature. By use of DSC analysis, the calculated T_g' for any known aqueous buffer formulation may be confirmed. In addition, DSC analysis can be used to uniquely identify and characterize a buffer formulation in terms of its measured T_g' . This same kind of analysis can also be applied to mixtures of buffer and biological cells.

Example 2

SUBSTITUTE SHEET

In Table 3 we predict the T_g' of a multicomponent mixture. By varying the individual components along the theme of monosaccharide/disaccharide/polymer, buffers are formulated with actual T_g' values measured by DSC analysis that correspond closely to the calculated value. Furthermore, by varying the individual component weight fractions in the solution (expressed as a w/v percent), and selecting polymers of desired average molecular weight, it is possible to design buffers that have a range of T_g' values within the useful temperature limits of -45°C to 0°C . It should be noted that the measured T_g' values in Table 3 are for 2.3:1 dilutions of buffer: water to mimic mixing the buffer with an aqueous suspension of cells. Furthermore, in a freeze-drying application (where water is removed from the system by either sublimation or evaporation), the starting T_g' will increase as the moisture content is reduced. Thus, although these buffers all have starting T_g' values in the range of -33.5°C to -21.25°C , the final dried product will have a T_g' considerably above these measured starting values. It is the final T_g' of the dried product that will determine the storage temperature limit for the dry material.

25 Example 3

In Figure 1 we described a hypothetical aqueous, partially crystalline system to illustrate the prediction from glass transition theory that the glass transition temperature of the amorphous phase (T_g') should rise as residual moisture decreases (or as % solids increases). From Figure 1 it can also be expected that the rise in T_g' is most rapid at low residual moisture levels (i.e., at about 10% residual moisture or less). In this example we demonstrate the practice of this concept with an aqueous

SUBSTITUTE SHEET

suspension of human red blood cells and a mixture of cryoprotectants, which is first frozen and then lyophilized to reduce the moisture content.

Packed human red blood cells were mixed in a 1 : 1.6 ratio by volume (1 part cells : 1.6 parts buffer) with a cryostabilizing aqueous buffer comprising the following components:

1.7 M glucose
15% (w/v) hydroxyethyl starch, 500,000 MW_{av}
10 3% (w/v) polyvinylpyrrolidone, 360,000 MW_{av}
Basic Buffer Salts (see Table 1).

The resulting mixture of red blood cells and cryoprotective buffers was analyzed by differential scanning calorimetry, and the onset of the Tg' transition for this mixture was observed to occur at about -38°C. The red blood cell and buffer mixture was then spread in thin layers in several Petri dishes and frozen at -40°C on the shelf of a Virtis lyophilizer (thin films were prepared to minimize temperature gradients that might form within thicker samples). After complete freezing, the samples were lyophilized at a constant shelf temperature of -38°C and pressure of 75 mTorr. At various time points separate samples were removed and weighed to determine the percent weight loss, from which it is possible to estimate the residual moisture content (the solids used to prepare the buffer are nonvolatile, so weight loss directly reflects the extent of removal of water vapor). The partially dried samples were also analyzed by differential scanning calorimetry to determine their glass transition temperature.

SUBSTITUTE SHEET

The results are shown in Figures 4-5. As expected, the sample T_g' increased with longer drying times, in a linear relationship (Figure 4). The drying cycle was stopped in this study after about 180 hours, at which time the sample glass transition temperature had risen to between $+2^{\circ}\text{C}$ to $+7^{\circ}\text{C}$. In Figure 5 our parallel weight loss measurements show that this rise in sample T_g' follows the loss of water vapor, as predicted by glass transition theory. As expected from the example in Figure 1, we observed a change in the rate of T_g' increase when the sample reached about 11% residual moisture (i.e., the slope of the curve changes). This confirmed our expectations that an incremental reduction in residual moisture at low moisture contents leads to a larger increase in the sample T_g' . Thus we expect that the residual moisture content of mixtures of biological cells and cryostabilizing compounds can be used to determine the product storage temperature and shelf life. The greatest advantage for ambient temperature storage will likely occur at low residual moisture content (i.e., at about 10% residual moisture or less).

This example illustrates the application of glass transition temperature theory to preservation of biological cells.

Example 4

Several of the buffer formulations listed in Tables 1-3 were used as cryoprotectants with fresh human red blood cells. Refrigerated packed red blood cells isolated from whole human blood collected in standard citrate-based anticoagulant were obtained from a blood bank. The cells were washed in dextrose-saline to remove the storage solution, and then 5 ml of washed red cells were mixed with 5.7 ml of each

SUBSTITUTE SHEET

buffer (all concentrations in Tables 1-3 are for the manufactured buffers, prior to mixing with cells). An additional 5.75 ml of each buffer was then added, with additional mixing. The buffer/red cell mixtures
5 were then frozen rapidly in a -80°C ethanol bath, and the frozen samples stored 24 hours at either -80°C or -38°C. Frozen samples were thawed in a 40°C water bath with agitation, and washed in a series of reconstitution buffers designed to return the cells
10 to isotonic conditions with minimal osmotic shock, and to remove any lysed cell debris. The thawed and washed cell suspensions were then subjected to analysis.

The post-thaw reconstitution procedure was
15 performed on each sample as follows:

1) Thawed red cells were removed from the 40°C water bath and 10 ml of 37°C reconstitution buffer (2000 mOsmol) was added. After addition of the first 10 ml, another 10 ml of 2000 mOsmol buffer was added
20 to the cells. The reconstitution buffers used in this procedure are based on a basic 300 mOsmol buffer recipe:

19% (w/v) PVP-10,000 MW
5 mM ATP
25 1.47 mM monobasic potassium phosphate
8.1 mM dibasic sodium phosphate
adjusted to pH 7.2

The above basic 300 mOsmol recipe was adjusted to either 2000 mOsmol or 1400 mOsmol by addition of
30 potassium phosphate.

2) After mixing-in the 2000 mOsmol buffer, the cells were further diluted by sequentially adding two 10 ml

SUBSTITUTE SHEET

aliquots of a 1400 mOsmol reconstitution buffer
(prepared according to the basic recipe above).

- 3) At this step a 200 microliter sample of cells is removed for measurement of cellular hemoglobin recovery (this data is presented in Tables 4-6 as the Cell Recovery at Reconstitution).
- 4) The remaining red cell suspension is centrifuged at 1500 xg for 5 minutes, and the supernatant discarded. The cell pellet is then suspended in 10 ml 1400 mOsmol buffer, and then another 10 ml of 1400 mOsmol buffer is added. This suspension is further diluted with two serial 10 ml aliquots of the basic 300 mOsmol buffer listed above, and the cells are again centrifuged and the supernatant discarded.
- 5) The final cell pellet is then suspended in two serial aliquots of the basic 300 mOsmol buffer containing PVP-10,000 MW. To this suspension two serial 10 ml aliquots of the following buffer are added to complete the osmotic step-down of the cells to isotonic conditions:
- 10% (w/v) PVP-2500 MW
 - 213 mM NaCl
 - 5 mM dibasic sodium phosphate
 - 5% glucose
 - pH 7.2 and 300 mOsmol

The remaining analyses shown in Tables 4-6 are performed on this final cell suspension.

The following in vitro parameters were measured for each sample:

SUBSTITUTE SHEET

- 1) Cell recovery at reconstitution (step 3 of the reconstitution protocol), measured by the recovery of cellular hemoglobin using a spectrophotometric assay.
- 2) Overall cell recovery after reconstitution of the cells in 300 mOsmol PVP-2,500 MW buffer, again measured by hemoglobin retention in the final cell pellet.
- 3) Osmotic stability of recovered cells in physiological saline (weakened cells lyse upon incubation in saline, and the released hemoglobin can be measured in the supernatant).
- 4) Red cell indices: Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), and Mean Corpuscular Hemoglobin Concentration (MCHC) are obtained from a Serono-Baker hematology analyzer.
- 5) The recovery of functional hemoglobin is measured spectrophotometrically, and expressed as the percent of oxyhemoglobin, methemoglobin, and hemichromes present in the sample.
- 6) Red cell deformability (Dlmax) is measured by ektacytometry, which measures the extent of cell elasticity when subjected to a viscous shear stress. The Dlmax expresses the maximum deformability, and can be expressed as a percent of the control Dlmax observed with fresh red cells.
- 7) An osmoscan profile is obtained by combining the ektacytometry with a continuous osmotic solution gradient, to evaluate deformability under hypertonic and hypotonic conditions.

SUBSTITUTE SHEET

- 8) The Relative Filtration Index (RFI) measures the ability of cells to traverse a filter containing a 5 micron pore size (normal human red cells are about 2x5 microns in size). This mechanical barrier
- 5 simulates circulatory stress encountered in the microvasculature.
- 9) Cell density is measured to assess the degree of cell swelling.

In Tables 4-6 we show the data obtained from

10 freeze-thaw experiments in which human red cells were frozen and then stored at either -80°C or -38°C for 24 hours, and then thawed, reconstituted to isotonic conditions, and analyzed. The cryoprotectant buffers #1-9 are the same as those listed in Table 2.

15 Duplicate cell samples (n=2) were evaluated for each buffer, and the mean values are reported in Tables 4-6. In each Table we also show the expected values obtained from fresh red cell samples analyzed as controls.

20 From the data in Tables 4-6 it can be seen that all of the freezing buffers tested confer significant cryostability to human red blood cells frozen and stored at -80°C, as measured by the percent of intact cells recovered at reconstitution. This protection

25 extends to initial cell recoveries for those samples stored as high as -38°C in the same buffer formulations. This result is consistent with the elevated calculated Tg' values (see Table 2) for these buffers. In addition, we noted that the PVP

30 polymer based buffers in Table 2 offered the advantage that very little cell aggregation occurred after thawing, in contrast to some of the buffer formulations reported in Table 1 that use different polymer species (for example, fructose-dextran

SUBSTITUTE SHEET

buffers were associated with cell aggregation, while fructose-PVP buffers exhibited no cell aggregation). Thus, although PVP and dextran can be used to elevate Tg', PVP is preferred since red cell aggregation is not observed after freeze-thawing in the presence of PVP.

The cryostabilizing properties of the buffers tested in Tables 4-6 at elevated temperatures of -38°C suggest that high temperature frozen storage of human red cells can be achieved. In Tables 4-6 we observed that in general the reconstituted red cells showed reduced cell deformability and filterability (the D_{lmax} and RFI values, respectively). In addition, elevated hemichrome levels were observed, as though some hemoglobin oxidation had occurred. These observations suggested that a further refinement of the cell permeant (i.e., the monosaccharide component) of these buffers might lead to improved cellular cryostability at elevated frozen temperatures. In the next example we added a highly membrane permeable polyalcohol, glycerol, to our buffer formulations.

Example 5

The history of human red cell cryopreservation has focused on the use of either high glycerol alone (40% w/v solution), requiring frozen storage at -80°C, or use of low glycerol (20% w/v solution), requiring frozen storage at -120°C in the vapor phase of liquid nitrogen. These very low storage temperatures are dictated by the low glass transition temperatures of aqueous glycerol solutions. From the principles of buffer design outlined herein, we have formulated low glycerol buffers containing carbohydrates and polymers that act as impermeant cryoprotectants that

SUBSTITUTE SHEET

also elevate the Tg' to a temperature range above -45°C.

In Tables 7-9 we demonstrate the effect of using multicomponent buffers containing glycerol or
5 propanediol as a highly permeant cryoprotectant, in combination with small sugars and various polymers. In these experiments human red blood cells were mixed in a 1:3 volume ratio (cells:buffer) with a particular buffer formulation, and then frozen and
10 stored at elevated freezing temperature (between -20° and -25°C). About 5 ml of fresh starting packed red cells were used. The frozen cells were thawed and reconstituted by a series of dilutions to return them to isotonic conditions, according to the following
15 protocol:

- 1) To the thawed cells two 5 ml aliquots of 2000 mOsmol reconstitution buffer (from Example 1) is added and mixed-in with the cells. This is followed by two 5 ml aliquots of 1400 mOsmol reconstitution
20 buffer (Example 1) to yield a diluted cell suspension.
- 2) The diluted cells are recovered by centrifugation and the supernatant is discarded. The cell pellet is suspended in 5 ml 1400 mOsmol buffer, then diluted
25 with another 3 ml of 1400 mOsmol buffer, then diluted again with two 5 ml aliquots of a 300 mOsmol dextrose-saline buffer (5% dextrose/0.9 % NaCl, w/v, adjusted to 300 mOsmol). At this point a 200 microliter sample is removed for the initial
30 measurement of cell recovery, as in Example 1.
- 3) The cells are again pelleted by centrifugation and the supernatant discarded. The cells are suspended in 5 ml commercial dextrose-saline (Baxter

SUBSTITUTE SHEET

Fenwal), and diluted with an additional 3 ml of commercial dextrose-saline. This final cell suspension is used for the remaining analyses in Tables 7-9.

- 5 The cell parameters measured in Tables 7-9 are as described in Example 1. In Table 7 we show the results of freezing red cells at high temperature (-22°C) in a series of buffers containing 25% v/v glycerol in the initial buffer formulation (the
10 starting buffer is diluted in the ratio of 1 volume packed red cells: 3 volumes buffer), so the final concentrations of buffer components in the frozen cell suspension is reduced. These conditions are also used in the experiments outlined in Tables 8 and
15 9.

- In Tables 7-9 a significant improvement in the cell deformability (DImax), osmoscan trace relative to fresh cells, and cell filterability index (RFI) are observed over the data shown in the preceding Tables
20 4-6. We propose that the combined advantages of the highly permeant polyalcohols with permeant and impermeant carbohydrates (both monosaccharides and disaccharides) and polymers having high component Tg values, allows for the recovery of thawed
25 reconstituted human red cells having excellent cell preservation qualities following frozen storage at temperatures as high as -22°C.

Example 6

- In this example we extend the application of glass
30 transition theory to freeze-drying of human red blood cells (freeze-drying can be achieved by direct sublimation, in which water vapor is removed from a

SUBSTITUTE SHEET

frozen sample whose temperature is held under the T_g' of the composition, or also by evaporative drying, herein defined as removal of water vapor from samples having temperature above the T_g').

5 In Table 10 we explored the use of cryostabilizing buffers containing 25% (v/v) starting concentration of glycerol as the permeant cryoprotectant, in combination with various carbohydrates (both mono- and disaccharides) and polymers. In these buffers we
10 focused on the application of hydroxyethylstarch (HES) as the polymer, having average molecular weights of 200,000 and 500,000 to elevate the buffer T_g' as predicted by glass transition theory. For comparison to the HES polymer (which has the added
15 advantage of USP pharmaceutical grade), we examined several starch hydrolysis products prepared from natural starches (i.e., potato starch), commonly used in food product formulations. The starch hydrolysis products examined included SPG20 and Paselli MD10.

20 For these experiments the human red blood cells were mixed with each of the buffers listed in Table 10, frozen, and then subjected to freeze-drying at a starting freeze-dryer shelf temperature of -25°C . We use a Virtis shelf lyophilizer in this process.
25 During the drying process the shelf temperature is ramped to $+4^{\circ}\text{C}$ without causing sample fluidity. This is consistent with glass transition theory, as we expect the T_g' of the initially frozen composition to increase as water is removed during the freeze-
30 drying. Hence the lyophilizer shelf temperature (and the sample temperature) can be raised as drying proceeds without causing sample fluidity and collapse. We monitored the amount of water removed from the samples by weight loss, and determined that
35 in all cases at least 90% of the water initially

SUBSTITUTE SHEET

present was removed (the solutes and cells being non-volatile). The dried samples were rehydrated and subjected to analysis as described in Tables 4-9.

In Table 10 each buffer formulation is designated by a number (i.e., SB 1965 -SB 1978). The components of each aqueous system, in addition to the 25% v/v glycerol present in each are shown (all percentages are percent w/v). As a control, buffer SB 1965 contains only 25% glycerol in 40 mM phosphate buffer, pH 7.4 (all other buffers in Table 10 are made in 40 mM phosphate pH 7.4). As further controls, SB 1966 and SB 1967 contain 15% w/v SPG20 or 7.5% Paselli, respectively, which are hydrolysis products of natural starches.

From Table 10 it can be seen that SB 1909 in particular confers significant protection to the dried red blood cells, as seen from the high osmotic stability in saline (88.8% of the cells survive exposure to physiological saline), normal red cell indices (MCV, MCH, MCHC), and improved deformability (Dimax = 92.6% of the value normally detected in fresh red cells). The filterability of these cells is also improved to some 50% of fresh red cell values. This sample was determined to have lost at least 90% of its moisture (i.e., contained 10% or less residual moisture) after drying. Buffer SB 1909 contains 25% (v/v) glycerol, .5% (w/v) glucose, 2.25% (w/v) lactose, and 7.25% (w/v) hydroxyethylstarch (HES-500 of 500,000 average molecular weight). This formulation illustrates the essential principles of the present invention: use of a highly membrane permeable component (glycerol), in combination with a membrane permeable monosaccharide (glucose), an impermeable disaccharide (lactose), and a high

SUBSTITUTE SHEET

molecular weight polymer (HES) to elevate the T_g of the multicomponent buffer.

In Figure 3 we show a representative osmoscan profile for the rehydrated red cells in sample SB 1909. The thin-line trace is the response observed with fresh control human red blood cells. The bold-type trace is the response of human red cells dried to at least 10% residual moisture at shelf temperatures ranging from -25°C and $+4^{\circ}\text{C}$, and then rehydrated to yield intact and viable red blood cells. From this osmoscan profile it can be seen that the maximum average cell deformability (DI_{max}), corresponding to the peak curve height, is very close (92.6% in Table 10) to that of fresh red cells. In addition, the overall curve profile is very near to that of fresh red cells. From our past experience with this method, we know that grossly rigid, damaged cells or cell debris yield a flat osmoscan profile, with a DI_{max} of 0.3 or less (data not shown). Figure 3 shows that the recovered rehydrated cells exhibit cellular rheology properties very similar to those of normal, untreated red cells, and this suggests that these cells retain the flexibility needed to survive circulation in the microvasculature following transfusion.

Example 7

In Table 11 we used fresh or one day-old human platelet concentrates to evaluate several polyvinylpyrrolidone (PVP) based cryostabilizing formulations. We combined different carbohydrates with the polymer, including monosaccharides and disaccharides. Hence the monosaccharide provides a membrane permeant cryoprotectant while the polymer and disaccharide are extracellular protectants. All

SUBSTITUTE SHEET

of the PVP-based formulations used with human platelet concentrates comprised a 10,000 average molecular weight PVP (PVP-10). We add these cryoprotectants directly to platelet concentrates in autologous plasma, which contains a buffering system, hence the formulations tested in Table 11 are prepared in 10 mM phosphate buffers, pH 7.2.

In Table 11 we show different cryoprotectant formulations comprising trehalose (tre), glucose (gluc), mannose (man), and/or lactose (lac), expressed in w/v percentages. We mix each buffer in a 1:1 proportion by volume with human platelet concentrate, and all w/v percents given in Table 11 refer to the final component concentration in the mixture of cryoprotectant solution and platelet concentrate. We examined cryoprotectant formulations containing one, two, or three primary cryoprotective components (i.e., carbohydrate or polymer alone, carbohydrate plus polymer, and carbohydrate mixtures plus polymer).

Samples of each mixture of cells and cryoprotectants were frozen at 10°C/minute in a controlled rate freezer to -60°C, and then transferred for storage to either a -80°C or a -40°C freezer. Samples were stored for 4-6 days. Each sample was then thawed in a 37°C water bath, diluted in phosphate buffered saline (PBS) to enable aggregometry analysis (the PVP must be diluted for proper assay conditions), and the cells recovered by centrifugation.

In this example and in the following examples herein, a standard set of cell quality assays are performed on the recovered cells. In Table 11 and our other platelet data herein, we show comparable

SUBSTITUTE SHEET

values obtained from control, untreated fresh platelet concentrates ("FRESH"). Our reported values are the mean \pm the standard error of the mean (SEM). These assays include:

- 5 1) Cell number recovery based on light microscopic examination and counting of the cells.
- 2) Cell morphology based on light microscopic examination and using the scoring method of Kunicki et al. (1975) Transfusion vol. 15, 414-421.
- 10 3) Aggregometry analysis using a Chronolog Lumi-Aggregometer to measure the normal platelet aggregation response to exogenous agonists. We measure the cell response to three treatments: adenosine diphosphate (ADP) + calcium, ristocetin (an
15 antibiotic that causes platelet aggregation), and collagen (the natural substrate that activates platelet aggregation).
- 4) Hypotonic shock response (HSR), stresses the platelets under hypotonic conditions to measure the
20 degree of cell flexibility. Healthy cells are able to withstand the shock and shrink to their normal cell volume upon return to isotonic conditions.

The data in Table 11 show that the formulations tested were comparable in preserving the measured in
25 vitro properties following frozen storage at -80°C for 4-6 days. In all cases the post-thaw assay values were about 50% of the control values observed in fresh, untreated platelet concentrates. Reduction in the hypotonic shock response is noted.

30 Example 8

SUBSTITUTE SHEET

In Table 12 we compared the properties of human platelets frozen and stored at -80°C , and then thawed and assayed as described in Example 1. We compared the trehalose/mannose/PVP-10 (TMP) formulation and the lactose/mannose/PVP-10 (LMP) formulation from Example 1 to dimethylsulfoxide (DMSO) or glycerol formulations reported in the blood banking literature. Due to the low glass transition temperature of 5% DMSO, cells stored in this medium are normally maintained at -80°C . We observed that the glycerol/glucose formulation resulted in the lowest cell recovery (this highlights the cell-type specificity of cryoprotectant formulation, as our red cell examples herein show the efficacy of low concentrations of glycerol (18-25% v/v) in red cell preservation). The mono- and disaccharide PVP buffers appear comparable in cell recovery to 5% DMSO at the selected storage temperature.

Example 9

In Table 13 we repeated the experiment shown in Table 12, but stored the platelet concentrates at -40°C . The TMP and LMP formulations both have $T_g' > -40^{\circ}\text{C}$, while the standard glycerol or DMSO solutions have $T_g' \ll -40^{\circ}\text{C}$ (for both the TMP and LMP formulations the measured T_g' by differential scanning calorimetry was about -31°C). The frozen samples were stored for 5-7 days. The resulting data show that the high T_g' TMP and LMP formulations yield better cell morphology and superior cell aggregation responses than the DMSO frozen cells. This suggests that frozen cell storage at temperatures much higher than conventional liquid nitrogen storage or -80°C mechanical freezer storage can be achieved by careful use of glass transition theory in cryoprotectant medium formulation.

SUBSTITUTE SHEET

Example 10

To extend the study in Example 9, we again prepared fresh, one day-old human platelet concentrates mixed 1:1 by volume with the

5 lactose/mannose/PVP- 10 formulation (LMP) as described in Example 1 (the solution is formulated as 10% w/v lactose, 5% w/v mannose, 10% w/v PVP-10,000 mM in 10 MW phosphate buffer, pH 7.2, and is diluted into an equal volume of platelet concentrate). We

10 then compared the ability of this formulation to preserve human platelets at elevated frozen storage temperatures as high as -25°C, relative to standard 5% DMSO. In this experiment four independent samples were prepared and frozen for each parameter (fresh

15 controls, DMSO, and LMP), and then stored at three different temperatures: -80°C, -38°C, and -25°C. In Table 14 we show the data obtained after the frozen platelets were stored for 7 days at each temperature, and then thawed and prepared for aggregometry

20 analysis by a single centrifugation step to remove most of the cryoprotectants. In Table 4 the aggregation response values are expressed as chart percentages. Morphology score and percent cell recovery are obtained as described in Example 7.

25 The data in Table 4 show that DMSO alone fails to adequately preserve measurable *in vitro* platelet responses, as well as cellular integrity, at elevated frozen temperatures. The high Tg' LMP formulation (Tg' approximately -31°C) yields improved functional

30 responses after storage at -38°C and -25°C relative to DMSO.

SUBSTITUTE SHEET

Table 1.

DSC Studies

Several buffer preparations were examined to determine their glass transition temperatures. A summary of the observed values is included in the following table:

<u>Buffer</u>	<u>Tg'</u> <u>Pre-</u>	
	<u>Tg'Observed</u>	<u>dicted</u>
10 5% Glucose, 10% lactose, 20% Dextran40	-26.5	-25.5
5% Glucose, 10% Lactose, 15% Dextran40	-25.5	-27.2
5% Glucose, 10% FCS, 20% Dextran40	-24.0	--
5% Glucose, 10% Lactose, 20% PVP40	-32.8	-31.2
5% Glucose, 10% Lactose, 15% PVP40	-31.0	-32.2
15 5% Glucose, 10% Lactose, 20% HES200	-30.0	-28.4
0-5M Glycerol, 7.5% BSA, 0.3M Glucose	-52.0	-51.0
0.5M Glycerol, 0.3M Glucose	Less than -60	-101

Legend to Table 1.

The predicted Tg' values were derived using Equation 1 for the individual components listed. Each buffer also contained buffer salts and metabolites as listed below (Basic Buffer Salts) whose contribution to calculated Tg' is ignored for the purpose of estimating Tg'. The measured Tg' values were obtained by DSC analysis of 2.3:1 dilutions of buffer:water to approximate the ratio of buffer:cell suspension used for cryostabilization of human red blood cells.

Basic Buffer Salts:

30 1.5 mM KH ₂ PO ₄	0.75 mM glutamine
8.1 mM Na ₂ HPO ₄	0.75 mM nicotinic acid
0.49 mM MgCl ₂	10 mM glutathione
10 mM inosine	pH = 7.4
5 mM adenine	

35 Dextran 40 = dextran 40,000 MW (av)

SUBSTITUTE SHEET

PVP 40 = polyvinylpyrrolidone 40,000 MW (av)
 FCS = fetal calf serum
 HES = hydroxyethyl starch 200,000 MW (av)
 BSA = bovine serum albumin

5

Table 2.

Three Component Buffers for
 Red Blood Cell Cryopreservation.

<u>Buffer Components@</u>		<u>Calculated+ Tg' (°C)</u>	<u>Measured* Tg' (°C)</u>
10	1) 5% mannose/10% lactose/ 20% PVP-40	-30.7	-30.0, -30.25
	2) 5% fructose/10% lactose/ 20% PVP-40	-30.8	N.D. (1)
	3) 5% glucose/10% lactose/ 20% PVP-40	-31.2	-30.5, -31.0
15	4) 5% mannose/10% trehalose/ 20% PVP-40	-31.1	N.D.
	5) 5% fructose/10% trehalose/ 20% PVP-40	-31.2	N.D.
20	6) 5% glucose/10% trehalose/ 20% PVP-40	-31.4	-32.1 (n=I)
	7) 5% mannose/10% sucrose/ 20% PVP-40	-31.8	N.D.
	8) 5% glucose/10% sucrose/ 20% PVP-40	-32.1	N.D.
25	9) 5% fructose/10% sucrose/ 20% PVP-40	-31.9	N.D.

@ All buffers include the Basic Salts listed in Table 1 herein. All percentages are (w/v) percents; PVP-40 is 40,000 average MW polyvinylpyrrolidone.

+ Calculated Tg' values from Equation 1 herein, taking buffer dilution into account.

* Measured Tg' values from DSC analysis on 2.3: 1 mixtures of buffer:water; separate values are shown from two independent measurements.

(1)N.D. = Not determined. From our data in Table 3 and buffers #1,3,6 above we find good correlation between calculated and DSC measured Tg' values.

SUBSTITUTE SHEET

Table 3.

Buffer Tg' Adjustment by Selection of Solute Components.

	<u>Buffer Components</u>	<u>Calculated Tg' (°C)</u>	<u>Measured Tg' (°C)</u>
5	10% glucose/10% lactose/ 20% PVP-40	-33.6	-33.0, -33.5
	10% glucose/10% lactose/ 20% HES-500	-31.2	-31.5, -31.75
10	5% glucose/10% lactose/ 20% PVP-40	-31.2	-30.5, -31.0
	5% glucose/10% lactose/ 10% dextran-40	-29.6	-30.25, -30.25
15	5% glucose/10% sucrose/ 20% dextran-40	-26.1	-26.0, -26.5
	5% mannose/10% sucrose/ 20% dextran-40	-25.8	-24.0, -23.75
	5% mannose/10% trehalose/ 20% dextran-40	-25.1	-23.75, -24.0
20	5% glucose/20% dextran-40	-22.4	-21.25, -21.25

All buffer components are expressed as (w/v) percentages, and are made in the Basic Salts formulation as outlined in Table 1. Calculated and measured buffer Tg' values are derived as outlined in

Table 1.

SUBSTITUTE SHEET

TABLE 4
 PROPERTIES OF RED CELLS STORED AT -80°C OR -38°C:
 BUFFERS 1-3

	Expected Values	Buffer #1 @ -80C n=2	Buffer #1 @ -38C n=2	Buffer #2 @ -80C n=2	Buffer #2 @ -38C n=2	Buffer #3 @ -80C n=2	Buffer #3 @ -38C n=2
Recov at Recon %	90% or greater	94.2	87.1	95.4	83.4	96.1	84.2
Overall Recov %	80% or greater	84.5	71.6	86.7	65.2	90.8	69.4
Osmotic Stability%	70% or greater	65.6	34.1	49.4	36.1	61.0	43.7
MCV (fl)	80-100	83.0	85.0	99.1	100.1	96.4	98.8
MCH (pg)	25-35	27.8	27.3	29.7	27.9	29.6	29.3
MCHC %	31-37	33.4	32.3	30.0	27.9	30.7	29.6
OXHb%	100	83.2	84.0	74.4	88.5	76.3	82.8
METHb%	0	0	0		0	0	
HEMICR	0%	15.8%	16.0%	25.6%	11.5%	23.8%	17.2%
DI max	0.500 or greater	0.505	0.385	0.472	0.425	0.493	0.421
% Contr DI	80% or greater	82.4	62.8	72.1	64.8	75.2	64.2
Osmoscan	Normal	Good but no Omin	Good but no Omin	Good but no Omin	Good but no Omin	Good but no Omin	Good but no Omin
RFI	0.650-0.700	ND	ND	0.350	0.310	0.384	0.309
%Contr RFI	80% or greater	ND	ND	37.2	34.3	42.6	34.2
Density g/ml	1.095 or greater	1.103	1.105	1.101	1.098	1.101	1.099

SUBSTITUTE SHEET

TABLE 5
 PROPERTIES OF RED CELLS STORED AT -80°C OR -38°C:
 BUFFERS 4-6

	Expected Values	Buffer #4 @ -80C n=2	Buffer #4 @ -38C n=2	Buffer #5 @ -80C n=2	Buffer #5 @ -38C n=2	Buffer #6 @ -80C n=2	Buffer #6 @ -38C n=2
Recov at Recon %	90% or greater	93.9	78.1	94.9	80.5	95.6	76.1
Overall Recov %	80% or greater	84.4	57.6	87.2	64.0	81.0	54.4
Osmotic Stability%	70% or greater	52.0	35.3	54.0	37.6	60.0	28.4
MCV (fl)	80-100	97.9	101.6	97.6	99.1	96.9	102.8
MCH (pg)	25-35	29.5	29.5	29.6	29.5	29.3	29.1
MCHC %	31-37	30.1	29.1	30.3	29.8	30.4	28.3
OXYHb	100%	76.3	82.3	75.8	78.8	80.4	76.9
METHb	0%	0	0	0	12.9	0	0
HEMICH	0%	23.7%	17.7%	24.2	8.3%	19.6%	23.1%
DI _{max}	0.500 or greater	0.509	0.432	0.467	0.419	0.512	0.422
%contr DI	80% or greater	77.8	65.9	71.2	64.0	78.2	64.5
Osmoscan	Normal	Good but no Omin	Good but no Omin	Abnormal	Abnormal	Good but no Omin	Abnormal
RFI	0.650- 0.700	0.314	0.268	0.364	0.319	0.442	0.317
%contr RFI	80% or greater	38.7	29.7	40.3	35.3	49.0	35.1
Density g/ml	1.095 or greater	1.098	1.097	1.100	1.097	1.098	1.100

SUBSTITUTE SHEET

TABLE 6
 PROPERTIES OF RED CELLS STORED AT -80°C OR -38°C:
 BUFFERS 7-9

	Expected Values	Buffer # 7 @ -80C n=2	Buffer #7 @ -38C n=2	Buffer #8 @ -80C n=2	Buffer #8 @ -38C n=2	Buffer #9 @ -80C n=2	Buffer #9 @ -38C n=2
Recov at Recon %	90% or greater	95.7	77.2	91.1	87.1	94.3	79.4
Overall Recov %	80% or greater	85.1	55.7	78.4	71.9	81.2	57.3
Osmotic Stability%	70% or greater	60.3	40.5	57.6	29.7	59.8	27.2
MCV (fl)	80-100	95.9	98.1	83.5	83.5	83.5	84.5
MCH (pg)	25-35	30.7	29.9	27.8	27.4	27.7	27.1
MCHC %	31-37	32.0	30.5	33.3	32.8	33.2	32.2
OXYHb	100%	70.9	81.5	88.0	88.1	88.9	84.6
METHb	0%	0	0	0	0.150	0	
HEMICH	0%	39.1%	18.6%	12.0%	11.7%	11.1%	15.4%
DI _{max}	0.500 or greater	0.518	0.446	0.478	0.362	0.449	0.344
%contr DI	80% or greater	79.0	68.0	78.2	59.1	73.3	56.2
Osmoscan	Normal	Good but no Omin	Abnormal	Good but no Omin	Abnormal	Good but no Omin	Abnormal
RFI	0.650- 0.700	0.479	0.334	ND	ND	ND	ND
%contr RFI	80% or greater	53.1	37.0	ND	ND	ND	ND
Density g/ml	1.095 or greater	1.099	1.102	1.103	1.105	1.106	1.088

SUBSTITUTE SHEET

TABLE 7

Summary of the results obtained with RBC stored at -22C in glycerol based cryobuffers. Note that the blood to buffer ratio for all the experiments is 1:3.

	Expected Values	25% Gly Stored at -22C for 15hrs	75% HES 25% Gly 15hrs at -22C	15% PVP +25% Gly 15hrs at -22C	7.5% PVP +25% Gly 15hrs at -22C	7.5% Lac +25% Gly 15hrs at -22C	7.5% Sucr +25% Gly 15hrs at -22C	5% Plur + 25% Gly 15hrs at -22C	7.5% Dex +25% Gly 15hrs at -22C
Recovery at Recon	90% or greater	98.1	95.5	94.7	89.6	97.6	95.2	97.8	93.5
Overall Recovery	80% or greater	91.7	74.1	85.5	71.4	93.5	90.0	89.5	74.7
Osmotic Stability	70% or greater	97.7	91.2	97.0	94.7	97.9	95.7	95.2	95.4
E	56.0 or greater	89.6	67.8	82.9	67.6	91.5	86.1	85.2	71.3
MCV (fl)	80-100	91.4	93.1	93.0	92.1	91.8	92.9	92.1	92.5
MCH (pg)	25-35	30.1	29.4	31.3	29.6	31.4	31.0	30.6	30.5
MCHC (g/dL)	31-37	33.0	31.6	33.6	32.2	34.3	33.3	33.1	33.0
Dlmax	0.500 or greater	0.657	0.645	0.636	0.630	0.643	0.613	0.639	0.662
%Control Dlmax	80% or greater	100	100	99.2	98.3	100	95.6	99.7	100
Osmoscan	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
RFI	0.700 or greater	0.940	0.860	0.840	0.800	0.900	0.880	0.840	0.860
%Control RFI	100	98.2	89.9	87.8	83.6	94.0	92.0	87.8	89.9
# of Samples	n=6	n=1	n=1	n=1	n=1	n=1	n=1	n=1	n=1

Abbreviations: Gly, Glycerol; Plur, Pluronic acid; Dext, Dextran 40; HES, Hydroxyethylstarch 250,000; Lac, Lactose; Sucr, Sucrose.

SUBSTITUTE SHEET

TABLE 8

Summary of the results obtained with RBC stored at -22C for 4 days. Note that the blood to buffer ratio for all the experiments is 1:3

	Expected Values	25%Gly Stored at -22C for 4days	75%HES 25%Gly at -22C for 4days	15%PVP +25%Gly at -22C for 4days	7.5%PVP +25%Gly at -22C for 4days	7.5%Lac +25%Gly at -22C for 4days	7.5%Sucr +25%Gly at -22C for 4days	5%Plur + 25%Gly at -22C for 4days	7.5%Dex +25%Gly at -22C for 4days
Recovery at Recon	90% or greater	92.9	94.9	95.3	92.4	92.7	92.3	93.7	90.6
Overall	80% or greater	87.4	82.3	87.4	78.8	88.8	86.3	84.5	65.5
Recovery	80% or greater	97.4	97.6	95.2	96.5	96.6	95.4	95.4	94.5
Osmotic Stability	70% or greater	85.1	80.3	83.2	76.0	85.8	82.3	80.6	61.9
E	56.0 or greater	91.9	92.5	93.6	93.4	92.2	92.0	91.7	92.0
MCV (fl)	80-100	31.0	30.4	32.1	30.2	30.9	30.6	30.3	30.0
MCH (pg)	25-35	33.6	32.9	34.4	32.3	33.5	33.2	33.0	32.6
MCHC (g/dL)	31-37	0.656	0.634	0.617	0.631	0.533	0.466	0.602	0.662
Dlmax	0.500 or greater	100	98.9	96.3	98.4	82.8	72.7	93.9	100
%Control	80% or greater	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Dlmax	Normal	0.907	0.840	0.800	0.860	0.740	0.760	0.860	0.840
Osmscan	Normal	94.8	87.8	83.6	89.9	77.3	79.4	89.9	87.8
RFI	0.700 or greater	n=1	n=1	n=1	n=1	n=1	n=1	n=1	n=1
%Control	80% or greater	n=1	n=1	n=1	n=1	n=1	n=1	n=1	n=1
RFI	80% or greater	n=1	n=1	n=1	n=1	n=1	n=1	n=1	n=1
# of Samples	n=6	n=1	n=1	n=1	n=1	n=1	n=1	n=1	n=1

Abbreviations: See Table 7

SUBSTITUTE SHEET

TABLE 9

Effects of 1,2-propanediol on the properties of cells stored at -22C. Table also shows a summary of the results obtained with 20% glycerol in combination with either lactose or 40K PVP. Note that the blood to buffer ratio for all the experiments is 1:3. Note also that some of the RBC were rapidly frozen at -80C and then stored at -22C for 15 hours.

	Expected Values	25% 1,2-propanediol, 15hrs at -22C	15%PVP + 25%Prp 15hrs at -22C	7.5%Lact +25%Prp 15hrs at -22C	7.5%Lact +20%Gly 15hrs at -22C.Slow freeze to -20C	7.5%Lact +20%Gly 15hrs at -22C. Fast frozen to -80C	15%PVP+ 20%Gly 15hrs at -22C. Slow freeze to -20C	15%PVP+ 20%Gly 15hrs at -22C. Fast freeze to -80C
Recovery at Recon	90% or greater	97.5	97.5	88.7	92.9	88.6	95.1	86.2
Overall Recovery	80% or greater	95.2	90.5	83.7	88.3	59.5	80.4	49.7
Osmotic Stability E	70% or greater	93.8	96.7	94.8	97.4	93.4	96.9	94.3
	56.0 or greater	89.3	87.5	79.4	86.0	55.6	77.9	46.9
MCV (fl)	80-100	94.7	94.2	90.1	84.6	91.9	86.6	90.8
MCH (pg)	25-35	31.0	30.1	31.4	30.3	25.8	28.3	25.0
MCHC (g/dL)	31-37	32.8	32.0	34.9	35.8	28.1	32.7	27.6
Dlmax	0.500 or greater	0.639	0.639	0.540	0.589	0.642	0.598	0.633
%Control Dlmax	80% or greater	99.7	99.7	84.2	92.6	100	94.0	99.5
Osmoscan	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
RFI	0.700 or greater	0.917	1.0	0.720	0.920	0.600	0.940	0.608
%Control RFI	80% or greater	93.6	100	73.5	96.0	62.8	98.3	63.6
# of Samples	n=6	n=1	n=1	n=1	n=1	n=1	n=1	n=1

Abbreviations : See Table 7

SUBSTITUTE SHEET

TABLE 10

	SB 1965	SB 1966	SB 1967	SB 1969	SB 1970	SB 1971	SB 1972	SB 1978- 20	SB 1978- 10
Comp (all w/ 25% Gly)	25% Gly alone	15% SPG 20	7.5% pasel	0.5 G 2.25L 7.25H 200K	.05G .65L 9.3H 500K	.15G 1.15L 8.2H 500K	0.5G 2.25L 7.25H 500K	2.0G 6.0L 12.0H 200K	1.0G 3.0L 6.0H 200K
%Dry	90	90	90	90	90	90	90	90	90
Rec	8.4	17.5	21.1	50.6	24.2	32.7	41.1	53.5	54.1
Osm	58	81	77.3	85.8	64.7	70.3	81.0	88.8	84.1
Stab									
MCV	93.2	102.1	96.4	104.8	98.0	96.1	98.6	94.5	96.4
MCH	10.4	29.3	14.1	29.2	27.0	28.0	30.8	31.0	30.2
MCHC	11.2	28.7	14.7	27.8	27.6	29.1	31.3	32.8	31.3
DI	-	80.7	60.7	85.3	-	-	-	92.6	88.5
Max									
Filter	-	-	-	-	-	-	-	50.0	50.0

Legend to Table 10

gly = glycerol

SPG20, Paselli = natural starch hydrolysis products

G = glucose

L = lactose

H 500K = hydroxyethyl starch 500,000 MW

H 200K = hydroxyethyl starch 200,000 MW

% DRY = % dryness by weight

(all numbers for individual buffer components are % w/v for solids and % v/v for glycerol)

SUBSTITUTE SHEET

TABLE 11

TABLE
Comparison of PVP buffers
Storage at -80°C for 4 to 6 days

N=4	FRESH	Single component		Two components		Three components		
		Trehalose 10%	PVP 10%	PVP 10% Gluc 0.6M	PVP 10% Man.2.5% Tre. 5%	PVP 10% Man.2.5% Lac. 5%		
REcovery SEM	100.00 0.00	34.63 4.60	42.25 3.17	60.27 16.23	43.25 6.71	44.83 2.47		
Morphology SEM	189.00 29.54	92.25 12.97	113.25 8.99	68.75 17.97	104.00 7.31	99.25 7.00		
ADP+ca SEM	70.93 6.12	31.15 2.94	32.83 4.02	5.33 2.74	23.78 3.76	27.23 3.61		
Collagen SEM	84.05 3.00	46.78 4.12	51.83 5.68	19.40 3.96	38.75 2.53	38.48 3.78		
Ristocetin SEM	83.10 3.91	32.00 6.39	36.23 1.60	30.53 11.23	26.38 4.72	29.18 3.30		
HSR SEM	41.30 11.05	4.93 3.06	7.53 3.87	5.47 2.39	2.15 1.86	1.18 1.02		

Recovery = Numerical recovery
Aggregation induced by ADP+Calcium, Collagen, Ristocetin.
HSR = Hypotonic shock response

SUBSTITUTE SHEET

TABLE 12

TABLE

Comparison DMSO or Glycerol buffers with Cryopropharm buffers at -80°C

N=4	FRESH	Cryopropharm buffers			
		DMSO 5%	Glyc. 5% Gluc. 0.6M	TMP 10%	LMP 10%
REcovery SEM	100.00 0.00	61.63 6.53	33.80 7.92	43.25 6.71	44.83 2.47
Morphology SEM	189.00 29.54	89.00 14.34	69.67 13.68	104.00 7.31	99.25 7.00
ADP+ca SEM	70.93 6.12	21.53 6.90	4.40 1.34	23.78 3.76	27.23 3.61
Collagen SEM	84.05 3.00	36.93 5.89	10.50 4.55	38.75 2.53	38.48 3.78
Ristocetin SEM	83.10 3.91	35.58 6.65	46.60 6.72	26.38 4.72	29.18 3.30
HSR SEM	41.30 11.05	2.08 1.04	10.53 6.21	2.15 1.86	1.18 1.02

SUBSTITUTE SHEET

TABLE 13

TABLE

Study of stability at different temperature

Storage at -40C

N=4	FRESH	Storage at -40C			
		DSMO 5%	TMP 10%	LMP 10%	
REcovery SEM	100.00 0.00	44.23 5.39	36.48 2.88	43.00 2.38	
Morphology SEM	189.00 29.54	38.00 1.27	73.75 4.52	65.50 4.37	
ADP+ca SEM	70.93 6.12	5.83 1.92	14.48 2.81	15.20 1.79	
Collagen SEM	84.05 3.00	14.13 3.64	33.03 3.79	33.10 3.51	
Ristocetin SEM	83.10 3.91	6.00 2.10	14.50 2.13	13.18 1.27	
HSR SEM	41.30 11.05	0.00 0.00	0.00 0.00	0.00 0.00	

SUBSTITUTE SHEET

TABLE 14

Storage at -80°C (n=4).

Cell Property	Fresh Controls	-80°C	
		DMSO	LMP
Cell Recovery	100	55.6 ± 5.3	45.2 ± 3.3
Morphology Score	184 ± 39	88 ± 19	91 ± 2
ADP Response	76.7 ± 4.8	27.9 ± 5.6	26.2 ± 4.7
Collagen Response	85 ± 3.8	37.2 ± 7.8	37.6 ± 4.9
Ristocetin Response	85.8 ± 4.2	33.3 ± 8.5	28.2 ± 4.3
HSR	51.2 ± 9.2	1.3 ± 1.1	1.6 ± 1.3

Storage at -38°C (n=4).

Cell Property	Fresh Controls	-38°C	
		DMSO	LMP
Cell Recovery	100	43.2 ± 7.1	44.4 ± 2.7
Morphology Score	184 ± 39	38 ± 1.7	63.3 ± 5.3
ADP Response	76.7 ± 4.8	5.1 ± 2.4	13.6 ± 1.5
Collagen Response	85 ± 3.8	11.3 ± 3.6	34.1 ± 4.5
Ristocetin Response	85.8 ± 4.2	5.9 ± 2.8	12.1 ± 1.2
HSR	51.2 ± 9.2	0	0

SUBSTITUTE SHEET

(Table 14, cont.)
Storage at -25°C (n=4).

	Fresh Controls	-25°C	
		DMSO	LMP
Cell Recovery	100	0	36.9 ± 3.3
Morphology Score	184 ± 39	0	20 ± 2.1
ADP Response	76.7 ± 4.8	0	3.2 ± 0.6
Collagen Response	85 ± 3.8	0	13.8 ± 0.9
Ristocetin Response	85.8 ± 4.2	0	0
HSR	51.2 ± 9.2	0	0

SUBSTITUTE SHEET

This page intentionally left blank

SUBSTITUTE SHEET

WHAT IS CLAIMED IS:

1. A process for freezing cells, cell membranes or cell-like materials comprising the steps of:
 - freezing an aqueous mixture comprising a
 - 5 plurality of cells, cell membranes or cell-like materials with a cryoprotective component;
 - wherein said aqueous mixture is characterized by a glass transition temperature of above about -60°C ;
 - said cryoprotective component comprising a first
 - 10 compound capable of permeating said cells, membranes, or cell-like materials and stabilizing said cells, membranes or cell-like materials for freezing;
 - and a second water-soluble compound impermeable to said cells, membranes or cell-like materials; the
 - 15 amount of said second compound in said mixture being in a weight fraction sufficient to overcome the predicted depression in the glass transition temperature of said mixture due to said first compound;
 - 20 wherein said freezing is performed by lowering temperature of said mixture to, or less than said glass transition temperature of said mixture, to form a frozen mixture.
2. A process according to Claim 1 further comprising
- 25 the step of drying said frozen mixture by sublimation or evaporation.
3. A process according to Claim 1 further comprising the steps of:
 - a. maintaining said frozen mixture at frozen
 - 30 storage temperatures at or below said glass transition temperature of said mixture;
 - b. thawing said frozen mixture;
 - c. optionally, reconstituting and/or washing said cells, cell membranes, or cell-like materials.

SUBSTITUTE SHEET

4. A process according to Claim 1 further comprising the steps of:
- a. drying said frozen mixture by either freeze-drying sublimation or evaporative drying to a dried,
5 solid mixture;
 - b. reconstituting and/or washing said dried solid mixture to recover rehydrated cells, cell membranes, or cell-like materials.
5. A process according to Claims 1, 2, 3, or 4
10 wherein said cryoprotective component comprises amorphous and/or partially crystalline polymers, or mixtures of different polymers.
6. A process according to Claim 5 wherein said polymers comprise natural and/or synthetic polymers.
- 15 7. A process according to Claim 6 wherein said natural and/or synthetic polymers comprise polymers having average molecular weights greater than 300.
8. A process according to Claim 7 wherein said polymer average molecular weights are between 10,000
20 and 500,000.
9. A process according to Claim 8 wherein said polymers are present in said solution in weight fraction percentages ranging from 1% up to the limit of solubility of said polymer in aqueous solution.
- 25 10. A process according to Claim 9 wherein said polymer weight fraction percents are in the range of 5%- 25%.
11. A process according to Claim 6 wherein said natural polymers comprise starch and/or starch
30 hydrolysis products.

SUBSTITUTE SHEET

12. A process according to Claim 11 wherein said starch comprises hydroxyethyl starch.
13. A process according to Claim 6 wherein said natural polymers comprise proteins, gelatin, dextran,
5 or other polymers derived from plant, animal, or microbial sources.
14. A process according to Claim 6 wherein said synthetic polymers comprise polyvinylpyrrolidone, polyethylene glycol, or other polymers derived from
10 organic synthesis.
15. A process according to Claims 1, 2, 3, or 4 wherein said component comprises carbohydrates or mixtures of different carbohydrates.
16. A process according to Claim 15 wherein said
15 carbohydrates or carbohydrate mixtures comprise monosaccharides and disaccharides.
17. A process according to Claim 16 wherein said monosaccharides and/or disaccharides are each present in a weight fraction percent between 0.01% up to its
20 solubility limit in aqueous solution.
18. A process according to Claim 17 wherein said weight fraction percent is between 0.5% and 20%.
19. A process according to Claim 16 wherein said monosaccharides are selected from the group
25 comprising 6 carbon hexose sugars.
20. A process according to Claim 17 wherein said hexose sugars comprise glucose, fructose, galactose, and mannose.

SUBSTITUTE SHEET

21. A process according to Claim 16 wherein said monosaccharides are selected from the group comprising 5 carbon pentose sugars.
22. A process according to Claim 19 wherein said
5 pentose sugars comprise ribose, xylose, arabinose, and ribulose.
23. A process according to Claim 16 wherein said disaccharides comprise maltose, lactose, sucrose, and trehalose.
- 10 24. A process according to Claims 1, 2, 3, or 4 wherein said component comprises polyalcohols.
25. A process according to Claim 24 wherein said polyalcohols comprise glycerol and 1,3- propanediol.
- 15 26. A process according to Claim 24 wherein said polyalcohols are present in concentrations between 0.1% and 40% by volume.
27. A process according to Claim 26 wherein said polyalcohols are present in the range of 5%-25% by volume.
- 20 28. A process according to Claims 1, 2, 3 or 4 wherein said component comprises aqueous mixtures of carbohydrates and/or polyalcohols and polymers.
- 25 29. A process according to Claim 28 wherein said mixture of carbohydrates and/or polyalcohols and polymers comprise membrane permeable and membrane impermeable components.

SUBSTITUTE SHEET

30. A process according to Claim 28 wherein said aqueous mixtures of carbohydrates and/or polyalcohols and polymers further comprise basic salts.
31. A process according to Claim 30 wherein said
5 basic salts comprise electrolytes, cofactors, metal cations, metabolites, buffer salts, vitamins, and protein or steroid hormones and growth factors.
32. A process according to Claim 30 wherein said aqueous mixtures of carbohydrates and polymers and
10 basic salts are characterized by a glass transition temperature of -60°C or higher.
33. A process according to Claim 32 wherein said glass transition temperature occurs between -45°C and $+45^{\circ}\text{C}$.
- 15 34. A process according to Claim 33 wherein said glass transition temperature is determined by thermal analysis.
35. A process according to Claim 34 wherein said thermal analysis measurements comprises differential
20 scanning calorimetry (DSC).
36. A process according to Claims 1, 2, 3 or 4 wherein said mixture of cells, cell membranes, or cell-like materials and cryoprotective component is characterized by a glass transition temperature of
25 -60°C or higher.
37. A process according to Claim 36 wherein said mixture has a glass transition temperature between - 45°C and $+45^{\circ}\text{C}$.

SUBSTITUTE SHEET

38. A process according to Claim 37 wherein said glass transition temperature is measured by differential scanning calorimetry.
39. A process according to Claims 1, 2, 3 or 4
5 wherein said cells comprise mammalian cells.
40. A process according to Claim 39 wherein said mammalian cells comprise human cells.
41. A process according to Claim 40 wherein said human cells comprise solid tissues.
- 10 42. A process according to Claim 40 wherein said human cells comprise peripheral blood cells.
43. A process according to Claim 42 wherein said peripheral blood cells comprise red blood cells, platelets, stem cells, and leukocytes.
- 15 44. A process according to Claims 1, 2, 3 or 4 wherein said cell membranes comprise mammalian cell membranes.
45. A process according to Claim 44 wherein said mammalian cell membranes comprise human cell
20 membranes.
46. A process according to Claim 45 wherein said human cell membranes comprise membranes of peripheral blood cells, including red blood cells, platelets, stem cells, and leukocytes.
- 25 47. A process according to Claims 1, 2, 3 or 4 wherein said cell-like materials comprise synthetic phospholipid membranes or vesicles.

SUBSTITUTE SHEET

48. A process according to Claim 47 wherein said vesicles comprise liposomes and liposome encapsulated hemoglobin.
49. A cryoprotectant medium for freezing or freeze-drying cells, cell membranes or cell-like materials comprising a first compound capable of permeating and stabilizing said cells, membranes or cell-like materials for freezing or freeze-drying, and a second water-soluble compound impermeable to said cells, membranes or cell-like materials, wherein the amount of said second compound in said medium is in a weight fraction sufficient to overcome the predicted depression in the glass transition temperature of said medium due to said first compound; said medium having a glass transition temperature of -60°C or higher.
50. A frozen-thawed composition prepared according to any of Claims 3 or 4.
51. A frozen composition prepared according to the process of Claim 1.
52. A freeze-dried composition prepared according to the process of Claim 2.
53. A composition according to Claim 52 wherein said step of drying is by sublimation.
54. A composition according to Claim 52 wherein said step of drying is by evaporative drying.
55. A medium according to Claim 49 wherein said first compound contains polyhydroxy groups.

SUBSTITUTE SHEET

56. A medium according to Claim 55 wherein said first compound comprises polyalcohols, monosaccharides, or mixtures thereof.
57. A medium according to Claim 49 wherein said
5 second compound contains polyhydroxy groups.
58. A medium according to Claim 58 wherein said second compound comprises disaccharides, polymers, or mixtures thereof.
59. A medium according to Claim 58 wherein said
10 polymers comprise natural starches, starch hydrolysis products, hydroxyethylstarch, or mixtures thereof.
60. A medium according to Claim 49 wherein said second compound comprises synthetic polymers.
61. A medium according to Claim 60 wherein said
15 synthetic polymers comprise polyvinylpyrrolidone.
62. A frozen-thawed composition according to Claim 50 comprising human cells and/or tissues.
63. A composition according to Claim 62 comprising human red blood cells.
- 20 64. A composition according to Claim 63 wherein said human red blood cells are characterized by normal red cell indices (MCV, MCH, MCHC), and/or osmotic stability in physiological saline of at least 70%, and/or an ektacytometric DI_{max} value of at least
25 0.500.
65. A composition according to Claim 62 comprising human platelets.

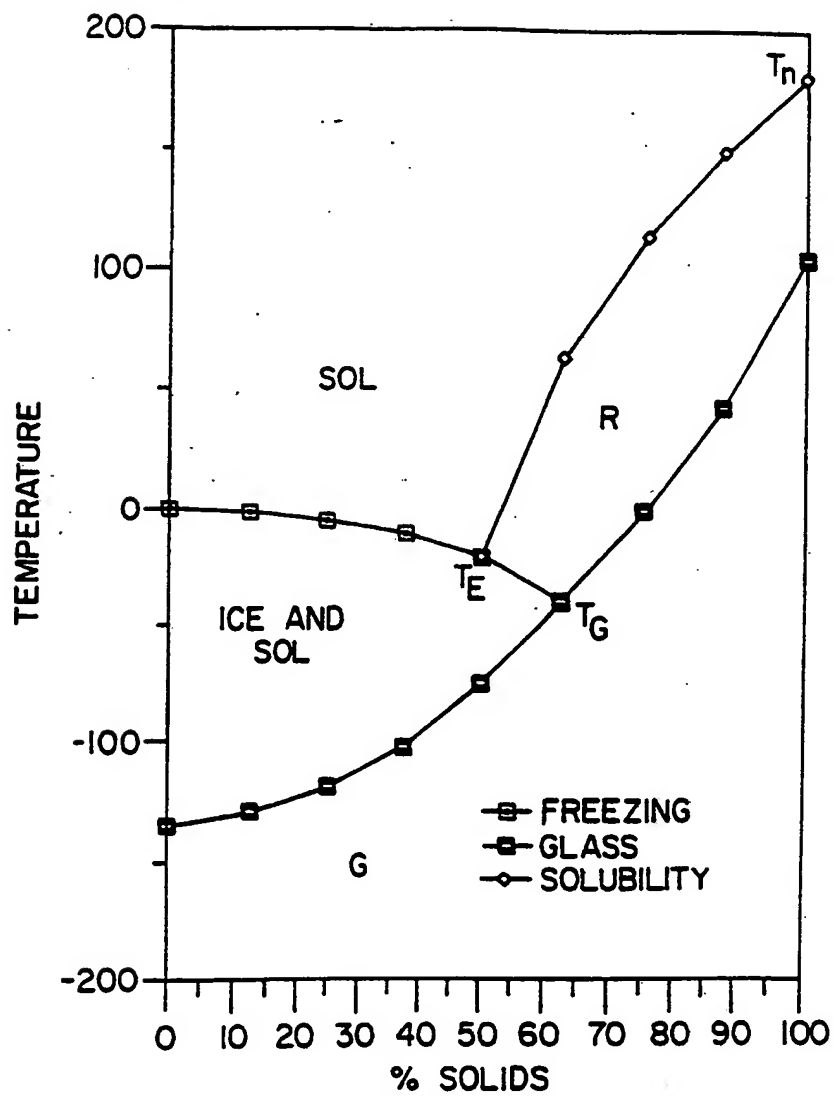
SUBSTITUTE SHEET

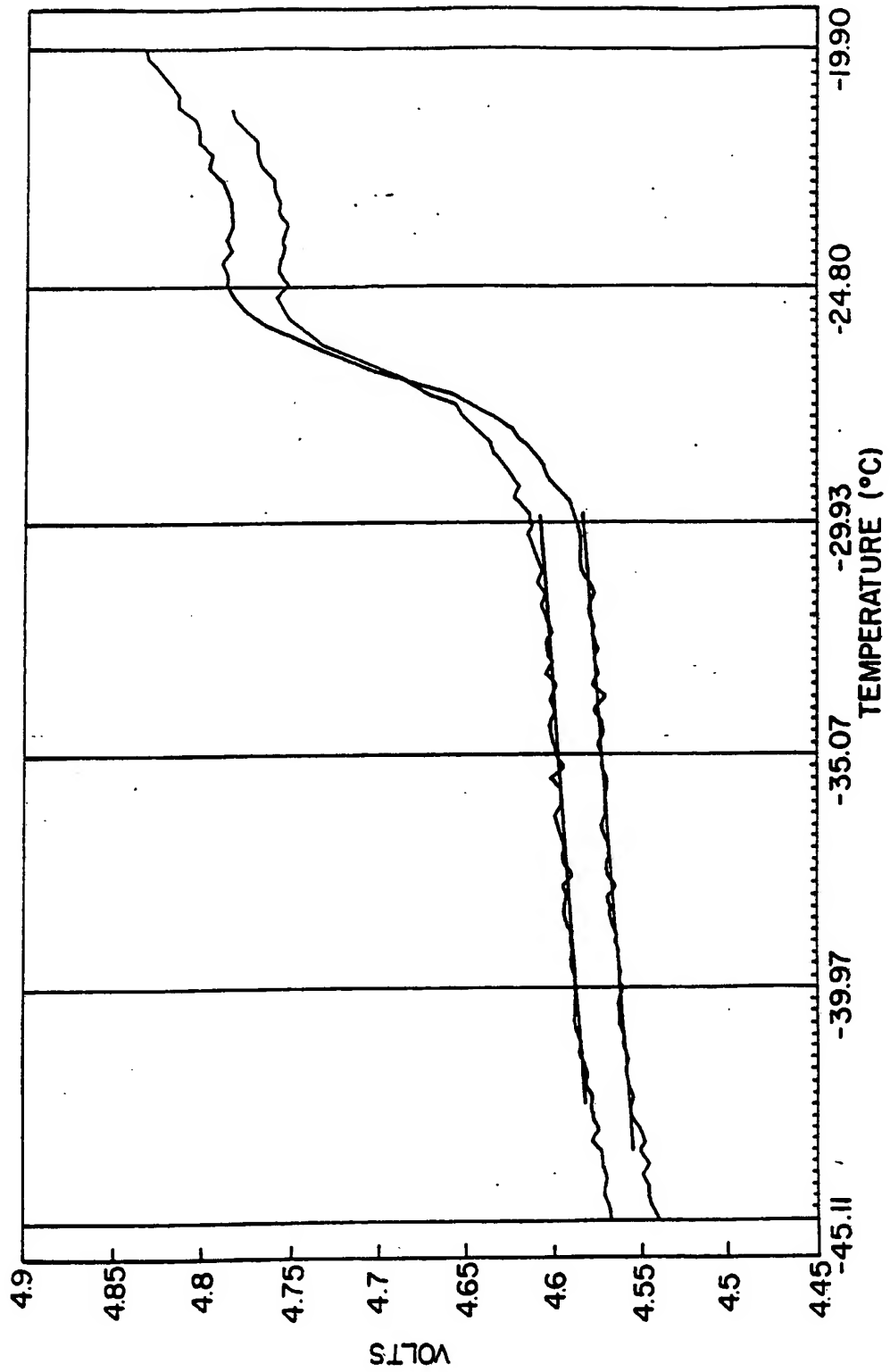
66. A composition according to Claim 65 wherein said human platelets are characterized by a measureable aggregometry response to platelet agonists.
67. A composition according to Claim 66 wherein said
5 agonists comprise collagen, ristocetin, and/or adenosine diphosphate and calcium.
68. A composition according to Claim 51 having a glass transition temperature of above about -60°C.
69. A composition according to Claim comprising human
10 cells and/or tissues.
70. A composition according to Claim 52 found by freeze-drying said composition in a frozen state in a temperature range of between about -45°C and 45°C.
71. A composition according to Claim 52 characterized
15 by a residual moisture content of 50% or less.
72. A composition according to Claim 52 characterized by a residual moisture content in the range of 1% to 30%.
73. A composition according to Claim 52 characterized
20 by a glass transition temperature that is above the glass transition temperature of said aqueous mixture.
74. A composition according to Claim 73 having said glass transition temperature of above about -40°C.
75. A frozen composition of human red blood cells or
25 platelets comprising an aqueous mixture of polyalcohols and/or carbohydrates and polymers, having a glass transition temperature above about -60°C.

SUBSTITUTE SHEET

76. A freeze-dried composition of human red blood cells or platelets comprising an aqueous mixture of polyalcohols and/or carbohydrates and polymers having a glass transition temperature of above about -60°C .
- 5 77. A freeze-dried composition according to Claim 76 having a glass transition temperature of above about -40°C .

SUBSTITUTE SHEET

**FIG. 1.****SUBSTITUTE SHEET**

**FIG. 2.****SUBSTITUTE SHEET**

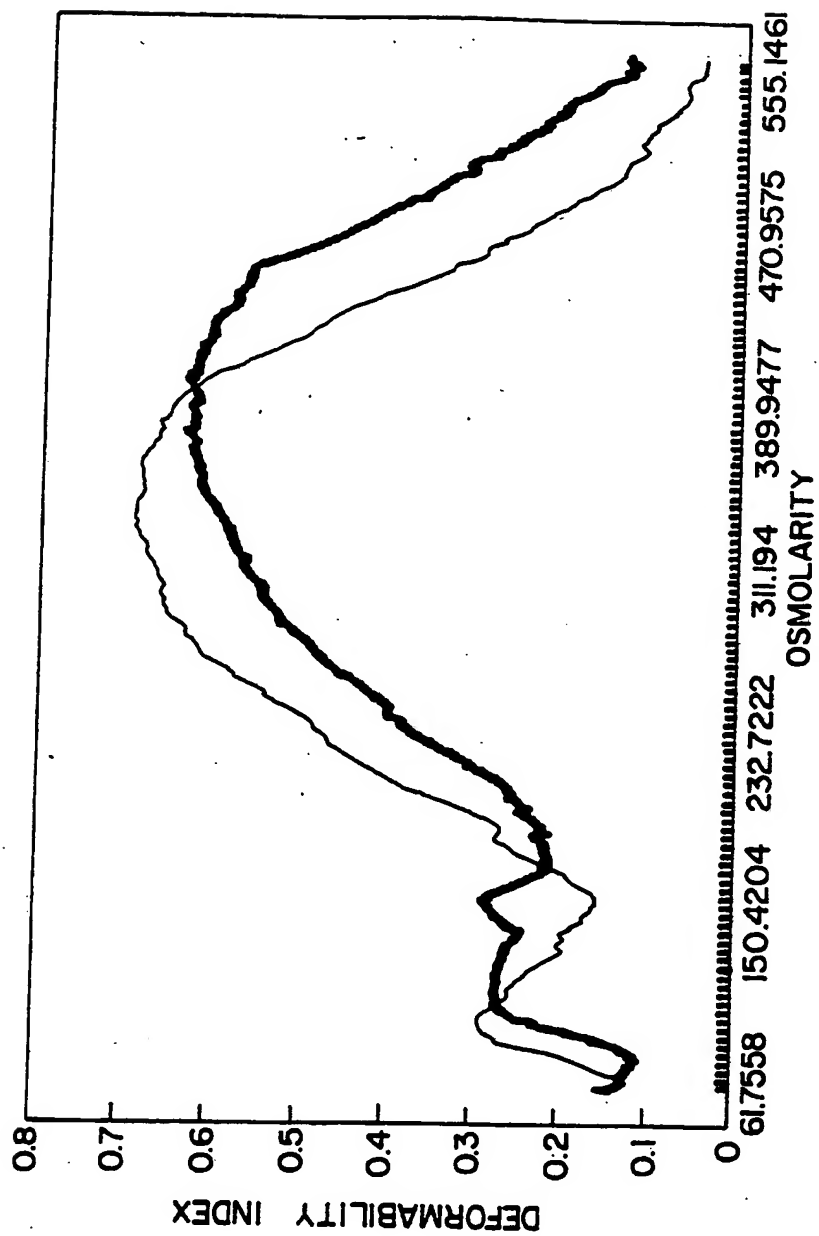
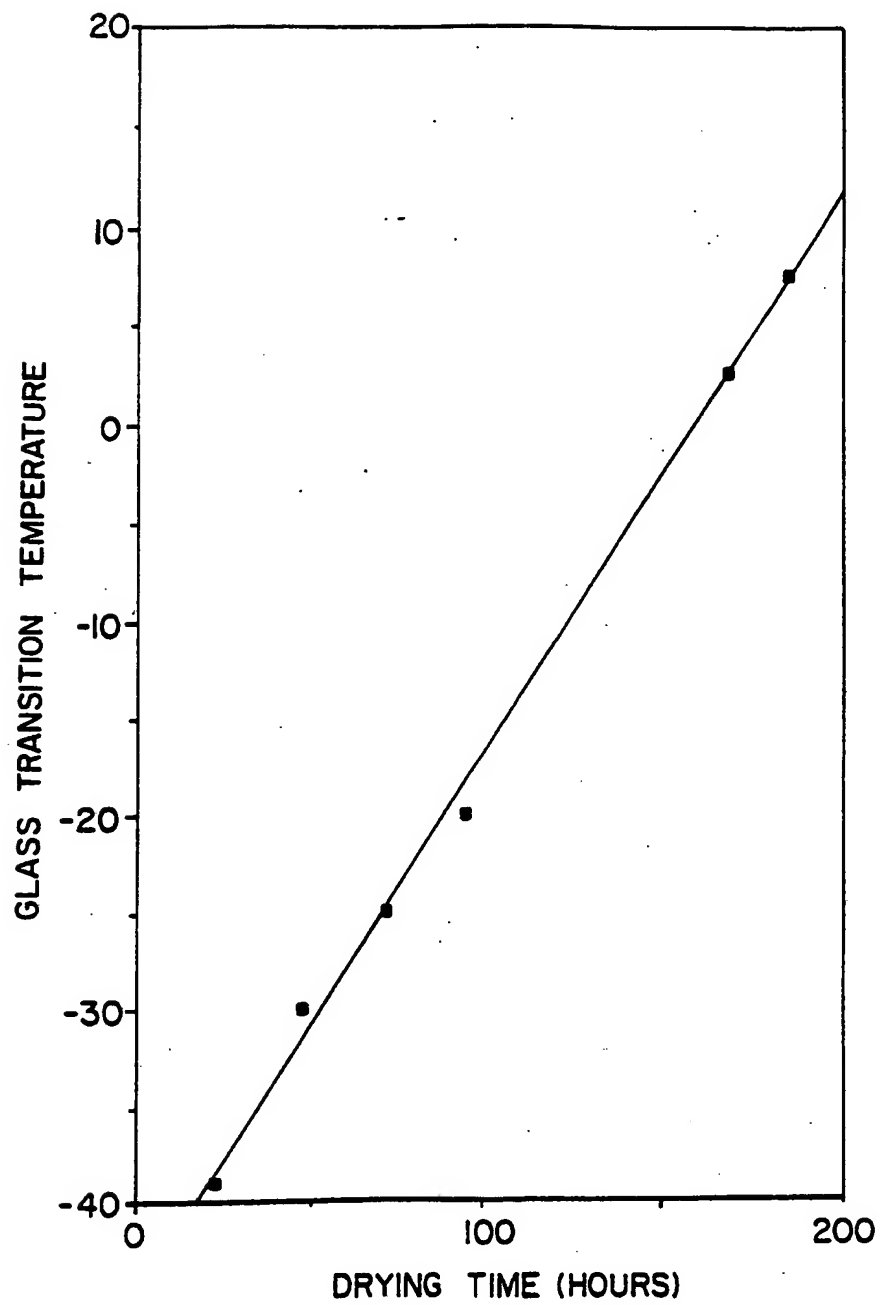
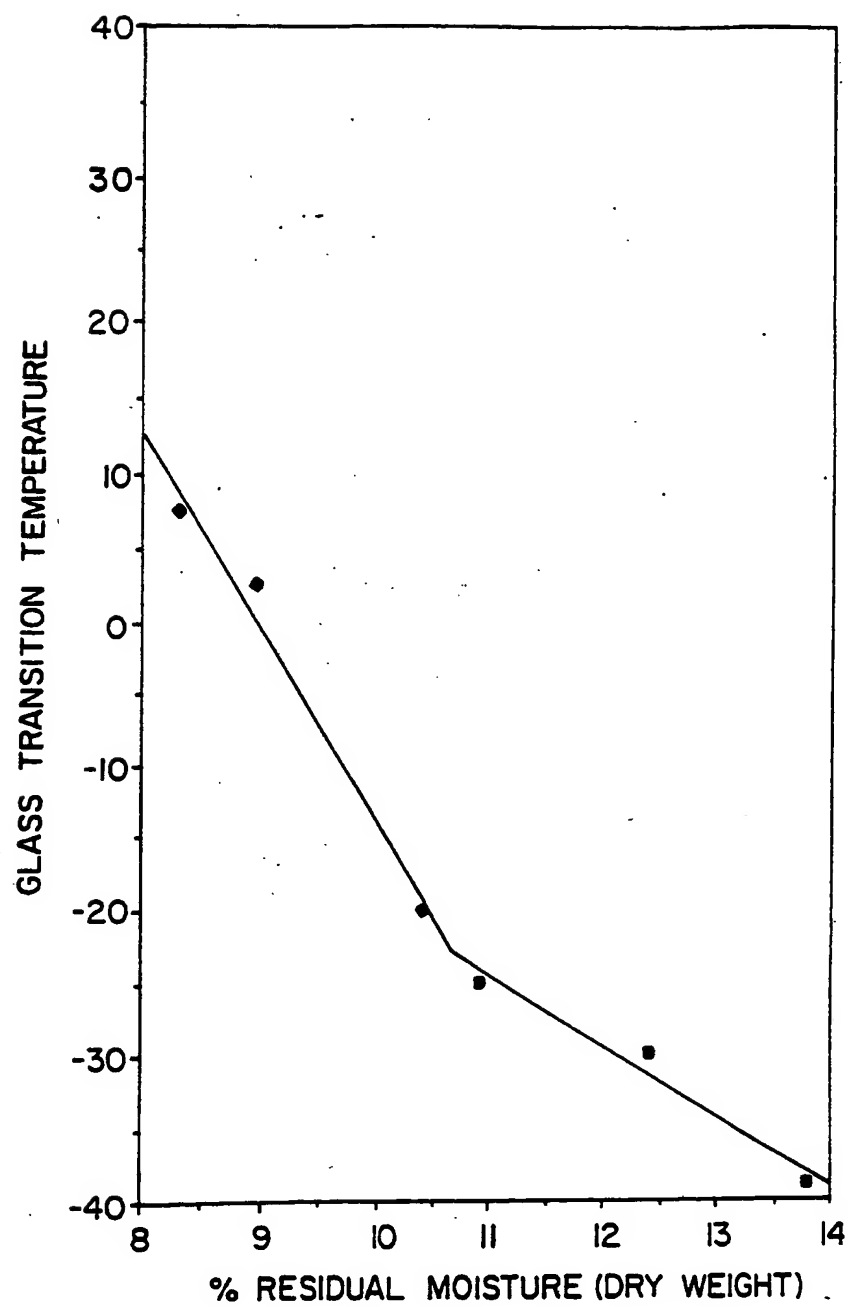


FIG. 3.

SUBSTITUTE SHEET

**FIG. 4.****SUBSTITUTE SHEET**

**FIG. 5.****SUBSTITUTE SHEET**

INTERNATIONAL SEARCH REPORT

L. national application No.

PCT/US93/00249

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :IPC(5): C12N 5/00; A01N 1/02

US CL :435/1, 2, 240.1; 252/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/1, 2, 240.1; 252/70

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	US, A, 5,045,446 (GOODRICH, Jr. ET AL) 03 SEPTEMBER 1991, SEE ENTIRE DOCUMENT.	1-48 — 49-77



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T"

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X"

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y"

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"A"

document member of the same patent family

Date of the actual completion of the international search

16 February 1993

Date of mailing of the international search report

5 MAR 1993

Name and mailing address of the ISA/
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. NOT APPLICABLE

Authorized officer

L. BLAINE LANKFORD

Telephone No. (703) 308-0196